

REMARKS

Claims 2, 5, 6, 8, 9, 11, 12, 14-38 and 53-65 are pending in this application. Claims 21, 22, 25 and 28-34 were withdrawn from consideration.

THE REJECTIONS

Claim rejection under 35 U.S.C. § 103(a)

Claims 2, 5, 6, 8, 9, 11, 12, 14, 23, 24, 26, 27, 35-38, 53, 56 and 57

The Examiner has maintained his rejection of claims 2, 5, 6, 8, 9, 11, 12, 14, 23, 24, 26, 27, 35-38, 53, 56 and 57 under 35 U.S.C. § 103(a) as being obvious over Kelly et al., "Intracellular adhesion molecule-1-deficient mice are protected against ischemic renal injury," J. Clin. Invest., 97(4):1056-1063 (1996) ("Kelly") in view of WO 93/04692 ("Kuberasampath") and Lefer et al., "Anti-ischaemic and endothelial protective actions of recombinant human osteogenic protein (hOP-1)," J. Mol. Cell. Cardiol., 24:585-593 (1992) ("Lefer"). The Examiner contends that Kelly discloses a role for ICAM-1 in mediating leukocyte-endothelial interactions, which may be involved in ischemic acute renal failure (ARF). The Examiner contends that Kuberasampath discloses OP-1 may be useful in modulating the tissue destructive effects of an inflammatory

response mediated, in part, by attachment of immune effector cells to endothelial cells. The Examiner further contends that Kuberasampath allegedly discloses that an inflammatory response by immune cell mediated tissue destruction can cause damage to the kidney. Finally, the Examiner contends that Lefer discloses that OP-1 has anti-adherent actions on polymorphonuclear leukocytes (PMNs). The Examiner concludes that it would have been obvious to one of ordinary skill in the art to administer an agent designed to block neutrophil-endothelial interaction to a mammal afflicted with ARF, as taught by Kelly, and to modify that teaching by administering OP-1, as taught by Kuberasampath and Lefer with a reasonable expectation of success. The Examiner states that one of ordinary skill in the art would be motivated to make the modification because agents designed to block neutrophil-endothelial interactions may be therapeutically effective in the prevention and treatment of acute renal failure and OP-1 is an agent designed to block neutrophil-endothelial interactions.

Applicants traverse.

Applicants respectfully submit that none of the cited references either alone or in combination, renders claims 2 and 53 (and claims depending therefrom) obvious. Applicants submit that

none of the references teach or suggest using an OP/BMP renal therapeutic agent to improve a standard marker of renal function in mammals afflicted with ARF as recited in the claims of the instant application. In fact, there is no teaching or suggestion in the cited references or from any other source that OP-1 inhibits ICAM-1 and therefore can be used to improve a standard marker of renal function in ARF.

The cited references do not teach or suggest that any anti-adherent action on immune cells will be useful in improving a standard marker of renal function in ARF. Kelly discloses that inhibition of ICAM-1 mediated neutrophil-endothelial interaction may play a role in improving ARF. However, one of ordinary skill in the art would not have expected that inhibition of any adherent action between immune cells and endothelial cells would improve a standard marker of renal function in ARF simply based on the effect of a single adhesion receptor molecule, ICAM-1. In fact, there are a large number of adhesion molecules that are sequentially activated during the multi-step process of transendothelial neutrophil migration (see Wagner et al., "Neutrophil Migration Mechanisms, with an Emphasis on the Pulmonary Vasculature," Pharmacological Reviews, 52:349-374 (2000) ("Wagner"), a copy of which is attached

herein as Exhibit A). As illustrated in Figure 1 of Wagner, depending on the particular stage of migration, different adhesion molecules are expressed on either neutrophils or endothelial cells and each of them exhibit different activity. Thus, one of skill in the art would not have expected that the effects of inhibiting an ICAM-1 mediated neutrophil-endothelial cell interaction would predict the effects of inhibiting any of the other adhesion molecules.

Furthermore, the cited references do not teach or suggest a nexus between OP-1 and ICAM-1. KuberaSampath discloses that OP-1 may have a role in modulating inflammatory responses that are mediated, in part, by attachments of immune cells to endothelial cells. However, there is no teaching or suggestion in KuberaSampath what OP-1 is modulating, i.e., through any specific adhesion molecule. Lefer does not remedy this deficiency. Again, there is no teaching or suggestion in Lefer what aspect of the adhesion process OP-1 is modulating on PMNs. Therefore, nothing in Kelly, KuberaSampath and Lefer teaches or suggests to the skilled worker to use OP-1 in ARF.

Applicants further submit that there was no teaching, suggestion or motivation in the state of the prior art to combine

these references. Kuberasampath does not teach or suggest a role for OP-1 in ARF. The broad teaching of inflammation having a role in damage in the kidney would not motivate one of ordinary skill in the art to connect OP-1 with treatments for ARF such as those described in Kelly as there are many types of kidney damage that include chronic and acute diseases and only a fraction of them may lead to ARF (see Brady et al; as cited by the Examiner). Furthermore, Kuberasampath and Lefer do not teach or suggest a role for an OP/BMP renal therapeutic agent in regulating ICAM-1. The anti-adherent role of OP-1 would not motivate one of ordinary skill in the art to combine these teachings with the treatments for ARF disclosed in Kelly as OP-1 could be acting through other adhesion molecules that do not play a role in ARF.

For all the above reasons, the teaching of Kelly, Kuberasampath and Lefer do not render the claims obvious. Accordingly, applicants respectfully request this rejection be withdrawn.

Claims 2, 15-20, 53-55 and 58-65

The Examiner has maintained his rejection of claims 2, 15-20, 53-55 and 58-65 under 35 U.S.C. § 103(a) as being obvious over the teaching of Kelly in view of Kuberasampath and Lefer and

further in view of Anderson et al., Chapter 275, in Harrison's Principles of Internal Medicine, (1980) ("Anderson") and Brady et al., Chapter 236, in Harrison's Principles of Internal Medicine, (1994) ("Brady").

Applicants respectfully maintain that at least for the reasons described above, nothing in Kelly, Kuberasampath and Lefer teaches or suggests the use of an OP/BMP renal therapeutic agent to improve a standard marker of renal function in ARF. Applicants submit that nothing in Anderson and Brady, either alone or in combination with any of the other documents, remedies this deficiency. Anderson discloses impaired cardiac output is a major cause of acute deterioration in renal function. Brady discloses that low cardiac output is a major cause of pre-renal acute renal failure. However, nothing in Anderson or Brady teaches or suggests a role for an OP/BMP renal therapeutic agent in regulating ICAM-1 and improving a standard marker of renal function in acute renal failure (ARF). Accordingly, applicants respectfully request that the Examiner withdraw this rejection.

Application No. 09/445,328

Reply to Pursuant to 37 C.F.R. 1.116 dated October 8, 2008

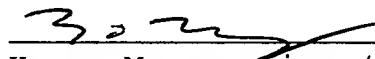
In response to Final Office Action of May 15, 2008

CONCLUSION

In view of the foregoing remarks, applicants request that the Examiner reconsider and withdraw all outstanding rejections and allow the pending claims.

The Examiner is invited to telephone applicants' representatives regarding any matter that may be handled by telephone to expedite allowance of the pending claims.

Respectfully submitted,

  
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# **EXHIBIT A**

# Neutrophil Migration Mechanisms, with an Emphasis on the Pulmonary Vasculature<sup>1</sup>

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**Abstract**—Leukocyte trafficking into pulmonary tissue and airspaces is a critical component of the host defense response. Activation and migration of polymorphonuclear leukocytes (PMNs) into lungs also contribute to inflammatory tissue injury and remodeling of tissue architecture. There have been considerable advances in our understanding of the mechanisms that control PMN adhesion and transendothelial migration (TEM). Mechanisms of migration unique to the lungs have been described with regard to the profile of adhesion molecules, cytokines, and chemokines elic-

ited during PMN emigration from blood vessels. This work reviews general mechanisms of TEM of PMNs and discusses the nature of PMN recruitment in several models of airway inflammation that illustrate how various stimuli elicit different responses. Pharmacologic manipulation of adhesive interactions between PMNs and endothelial cells is a current area of research aimed at developing pharmacologic agents to control inflammation during pulmonary and other inflammatory diseases. A summary of some of these agents and their actions is presented.

## I. Introduction

Knowledge of the mechanisms of leukocyte migration has expanded greatly in recent years. Whereas only in the last decade the first adhesion molecule was being characterized, today our understanding includes molecular descriptions for numerous adhesive receptor-ligand interactions, discovery of novel families of chemotaxins and cytokines, and the appreciation for the pivotal role adhesion pathways play in diseases from AIDS and atherosclerosis to cancer and inflammatory syndromes. Although these research efforts have clarified some issues, they also have illuminated new and perhaps difficult challenges. Indeed, the current literature on leukocyte transendothelial migration (TEM)<sup>3</sup> portrays a complex phenomenon with many mechanisms and points of con-

trol that depend on the site of migration, the cell type, the initial inflammatory stimuli, and the presence and absence of several cellular and soluble mediators. The simple paradigm of rolling/adhering/diapedesis still holds true. However, the number of variables involved in our understanding of the process has increased dramatically.

This review will first describe the general mechanisms of TEM and the current understanding of the adhesion molecules, cytokines, and chemokines that drive leukocyte migration with an emphasis on polymorphonuclear leukocytes (PMNs; neutrophils). Also discussed are the interactions among soluble mediators that together determine the outcome of the adhesive/migratory process. Next, the special case of pulmonary neutrophil migration will be used to illustrate the dependence of the form of migration on the specific inflammatory stimuli. A summary of these observations in lung support the hypothesis that different adhesion molecule pathways are invoked by a qualitative difference in the inflammatory cytokines and chemokines present. Last is a brief discussion of current clinical and experimental strategies for pharmacologic interventions that target the adhesive process.

## II. General Mechanisms of Transendothelial Neutrophil Migration

Circulating leukocytes can migrate from vessels into tissues under both normal and pathologic circumstances. It is well accepted that leukocyte migration

<sup>3</sup> Abbreviations: TEM, transendothelial migration; COX, cyclooxygenase; CINC, cytokine-induced neutrophil chemoattractant; EC, endothelial cell; E-selectin, endothelial cell selectin; fMLP, formyl-methionyl-leucyl-phenylalanine; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; ICAM, intercellular adhesion molecule; I domain, inserted domain; IL, interleukin; LFA-1, lymphocyte-associated function antigen-1; LIX, LPS-induced CXC chemokine; LPS, lipopolysaccharide, endotoxin; LRI, leukocyte response integrin; L-selectin, leukocyte selectin; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; Mac-1, macrophage antigen-1; MIP-2, macrophage inflammatory protein-2; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NIF, neutrophil inhibitory factor; NSAID, nonsteroidal anti-inflammatory drug; PAF, platelet-activating factor; PECA, platelet-endothelial cell adhesion molecule; PG, prostaglandin; PMN, polymorphonuclear leukocyte neutrophil; P-selectin, platelet selectin; PSGL-1, P-selectin glycoprotein ligand-1; RGD, arginine-glycine-aspartic acid; SLE<sub>x</sub>, sialyl-Lewis<sub>x</sub>; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VCAM, vascular cell adhesion molecule; VLA, very late antigen; ZAS, zymosan-activated serum.

from the vasculature occurs by a multistep process, dictated by the sequential activation of adhesive proteins and their ligands on both leukocytes and endothelial cells (ECs) (Lawrence and Springer, 1991; von Andrian et al., 1991; Konstantopoulos and McIntire, 1996). Monocytes, lymphocytes, and PMNs all migrate by these similar, sequence-dependent mechanisms but differ in their responses to chemotactic and inflammatory signals, particularly in their qualitative and quantitative expression of adhesion molecules (Dransfield et al., 1992; Springer, 1994; Ager, 1996; Li et al., 1996). Initiation of migration begins with the "capture" by the vessel wall of PMNs from flowing blood, and this is followed by their "rolling" along the vessel wall. This process of margination is a normal behavior of circulating PMNs. Only after appropriate stimuli are present do rolling leukocytes become firmly adhered to ECs and are thus positioned for migration from the blood vessel into tissue parenchyma.

#### A. Capture and Rolling

Both the capture, or initial tethering and removal of PMNs from the flowing blood, and their rolling along the vessel wall is due to the reversible binding of transmembrane glycoprotein adhesive molecules called selectins, which are found on both PMNs and ECs (Bevilacqua and Nelson, 1993; Albelda et al., 1994; Luscinskas and Lawler, 1994; Crockett-Torabi and Fantone, 1995; Tedder et al., 1995a). Selectins have a calcium-dependent lectin domain on the extracellular NH<sub>2</sub> terminus that is attached to an epidermal growth factor-like domain and then to a number of short consensus sequences. A short, intracellular domain is linked to signal transduction proteins (reviewed in Crockett-Torabi and Fantone, 1995; Crockett-Torabi, 1998). Selectin-type adhesive proteins are found on most cell types of hematopoietic origin and on ECs of blood and lymph vessels.

**1. Leukocyte Selectin.** Intravital microscopic analysis of the microvascular circulation in normal tissue offers visual evidence of the transient "stick and release" behavior of PMNs rolling along the vessel wall. In non-inflamed tissues, the tenuous association of PMNs within postcapillary venules can be blocked by treatment with antibodies to leukocyte selectin (L-selectin) (Mel-14, LAM-1, CD62L) on circulating PMNs (Spertini et al., 1991; von Andrian et al., 1991). Constitutive expression of L-selectin is greatest on PMNs newly released from bone marrow compared with older, circulating PMNs (Matsuba et al., 1997). Loss or shedding of L-selectin from the PMN surface is due in part to metalloprotease activity, which results in rapid accumulation of bioactive L-selectin in the blood (Kishimoto et al., 1995). Autoproteolysis of L-selectin can occur after exposure to various inflammatory mediators such as lipopolysaccharide (LPS; endotoxin) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), but it may also occur from normal rolling interactions with the vessel wall. For example, metallo-

protease inhibitors significantly reduce rolling velocity and cleavage of L-selectin on PMNs *in vitro*, suggesting that L-selectin is routinely shed from circulating PMNs during normal, nonpathologic conditions (Walchek et al., 1996). Thus, the longer a PMN has been in the circulation and interacting with the vessel wall, the more L-selectin it has lost to transient binding and cleavage. Replacement of L-selectin on circulating PMNs has not been demonstrated, and a low expression level is associated with apoptosis and may be a signal for the removal of PMNs from the circulation (Matsuba et al., 1997). High plasma levels of soluble L-selectin that can occur during infection may inhibit PMN rolling at non-inflamed sites. Bioactive, soluble L-selectin can bind to endothelial ligands and block their interactions with PMN-borne L-selectin (Schleiffenbaum et al., 1992; McGill et al., 1996; Ohno et al., 1997a).

Although not fully characterized, the corresponding endothelial ligand of PMN L-selectin is a member of a group of sialomucin oligosaccharides that share affinity for selectins expressed on platelets, lymphocytes, and monocytes (Varki, 1997) (Fig. 1). Studies *in vitro* demonstrate an endothelial ligand for PMN L-selectin that is induced by LPS or cytokine exposure of endothelial monolayers (Spertini et al., 1991). In addition, L-selectin-dependent rolling of PMNs occurs in noninflamed tissues, and this suggests the existence of a constitutively expressed endothelial counterpart to L-selectin (von Andrian et al., 1991; Walchek et al., 1996).

The best characterized ligand for L-selectin is CD34, which is found on high vein ECs and binds selectively to L-selectin of lymphocytes (Tedder et al., 1995; Ager, 1996). CD34 and related molecules are long protein chains heavily modified with *O*-linked sugar and sialyl groups and have variable binding affinities to all selectins in nonflow systems *in vitro*. The endothelial ligand for L-selectin is believed to be a fucosylated variant of CD34 (Tedder et al., 1995; Krause et al., 1996).

**2. Platelet Selectin.** At least two endothelium-bound selectins, platelet selectin (P-selectin) and endothelial cell selectin (E-selectin), can facilitate PMN-EC adhesions. These selectins are expressed only when appropriate inflammatory stimuli are present. P-selectin (granule membrane protein-140; CD62P) is stored intracellularly in Weibel-Palade bodies of ECs and in  $\alpha$ -granules of platelets (Malik and Lo, 1996). Within minutes of exposure of ECs to inflammatory mediators such as complement products, oxygen-derived free radicals, or various cytokines, P-selectin is mobilized to the cell surface where it can interact with its PMN counterpart, P-selectin glycoprotein ligand-1 (PSGL-1; CD162). Monocytes and platelets also possess PSGL-1 and can bind to P-selectin on activated ECs. Like CD34, the PSGL-1 protein is modified with *O*-linked sialic acid and other sugar groups. It consists of a disulfide-bonded homodimer on the PMN surface and is capable of binding two P-selectin ligands simultaneously (McEver and Cummings, 1997).

## Neutrophil - Endothelial Cell Ligands

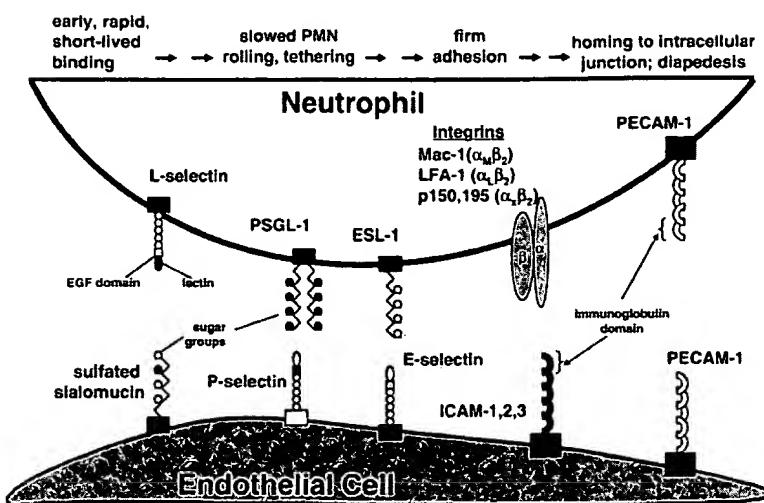


FIG. 1. Schematic summary of adhesion molecules and respective ligands involved in TEM of neutrophils. EGF, epidermal growth factor; ESL-1, E-selectin ligand 1.

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PSGL-1 is uniformly distributed on quiescent, rolling PMNs. L-selectin binding occurs first and is more rapid and short-lived than P-selectin binding (Ley et al., 1995). Binding to P-selectin is characterized by longer PMN-EC associations, slower rolling velocities, and eventual tethering of PMNs to the vessel surface (Alon et al., 1997; Davenpeck et al., 1997). As with L-selectin binding however, P-selectin-PSGL-1 interaction is short-lived and reversible if additional adhesive events are not soon invoked (Lawrence and Springer, 1991; Finger et al., 1996; Davenpeck et al., 1997). In the presence of appropriate inflammatory stimuli, P-selectin binding is accompanied by a rapid redistribution of PSGL-1 to uropods on activated PMNs and may signal the transition from rolling to capture (Bruehl et al., 1997).

**3. Endothelial Cell Selectin.** A second endothelial-borne selectin, E-selectin (ELAM-1, CD62), is not stored but requires gene transcription for expression. Peak expression and activity in ECs in vitro is 4 to 6 h after exposure to inflammatory cytokines (Klein et al., 1995; Scholz et al., 1996). E-selectin can support rolling and tethering of PMNs in a fashion similar to P-selectin (Lawrence and Springer, 1994). Thus, its role in inflammation may be to maintain PMN rolling after P-selectin has been down-regulated (Malik and Lo, 1996; Yang et al., 1999a). Like L- and P-selectins, E-selectin binds in vitro to a variety of sialic acid- and fucose-containing glycoproteins, as well as to sulfated glycosaminoglycans such as heparan sulfate. However, the in vivo ligand for E-selectin may be a unique mucin modified with *N*-linked sugars that has been characterized in murine cells called E-selectin ligand 1 (Levinowitz et al., 1993; Steegmaier et al., 1995; Willmroth and Beaudet, 1999). Another sialylated ligand for E-selectin has been described in bovine T cells and is distinct from E-selectin

ligand 1 (Walchek and Jutila, 1993). Human analogs for either murine or bovine ligands have not been reported.

To summarize briefly, L-selectin (rolling on ECs) and P-selectin (capture) work cooperatively to initiate the migration process during inflammation (Ley, 1996; Alon et al., 1997; Davenpeck et al., 1997). Selectins and their PMN ligands are either constitutively expressed or stimulated to mobilize to the cell surface. These reversible selectin-ligand interactions allow time for PMNs to associate with ECs and integrate and respond to stimuli presented on the endothelial surface. Characterization of selectin-mediated capture and rolling has been done predominately in systemic vessels (Lawrence and Springer, 1991; von Andrian et al. 1991; Ley et al., 1995; Davenpeck et al., 1997). However, in the pulmonary circulation, recent intravital microscopic studies show different selectin involvement during PMN-EC interactions, and these will be discussed in the next section (Kuebler et al., 1997; Yamaguchi et al., 1997).

#### B. Firm Adhesion

The initiating signal for the next step of TEM, that of firm adhesion, is postulated to be either a receptor-mediated event in response to an inflammatory cytokine or an event propagated from signals from activated selectins. Cytoplasmic domains of bound and activated L-selectin and PSGL-1 are linked to signal transduction pathways that lead to integrin activation in PMNs (Crockett-Torabi and Fantone, 1995; Simon et al., 1995, 1999; Zimmerman et al., 1996a; McEver and Cummings, 1997; Williams and Solomkin, 1999). Thus, selectins may function to promote the orderly transition to the adhesion process by invoking integrin expression pathways in a timely, sequential manner that ensures successful migration.

**1. Integrins.** Integrins are a group of heterodimeric transmembrane glycoproteins found on PMNs and other hematopoietic cells that mediate cell-cell and cell-extracellular matrix adhesions (Hynes, 1992; Luscinskas and Lawler, 1994). All integrins comprise one  $\alpha$ - and one  $\beta$ -subunit, which together form an extracellular ligand binding site. Cytoplasmic tails of integrins provide phosphorylation sites and linkages to cytoskeletal proteins involved in signal transduction (Alpin et al., 1998). There are 8 different  $\beta$ -subunits ( $\beta_1$ – $\beta_8$ ) that associate with one of 16  $\alpha$ -subunits to form at least 23 known receptors in a variety of cells, including lymphocytes, leukocytes, and platelets. Integrins are capable of mediating cell-cell binding but also are involved in cell interactions with extracellular proteins such as laminin, fibronectin, vitronectin, and fibrinogen, to name a few.

PMN binding to activated endothelium is mediated primarily by two integrins that consist of  $\beta_2$ - (CD18) subunits. These are macrophage antigen-1 (Mac-1;  $\alpha_M\beta_2$ ; CD11b/CD18) and lymphocyte-associated function antigen-1 (LFA-1;  $\alpha_L\beta_2$ ; CD11a/CD18) (Fig. 1). LFA-1 is the predominate integrin used for lymphocyte emigration (Li et al., 1996). The low level of basally expressed LFA-1 on PMNs is unaltered by activators or stimuli. However, both LFA-1 and a third CD18 integrin, p150,95 (CD11c/CD18;  $\alpha_X\beta_2$ ), can promote PMN trafficking under certain conditions. A fourth member of the  $\beta_2$ -integrin family,  $\alpha_D\beta_2$ , has been recently described as a critical mediator of eosinophil adhesion. This integrin has yet to be reported on PMNs (Grayson et al., 1998; van der Vieren et al., 1999).

Mac-1 has emerged as the more critical CD18-containing integrin in most models of PMN-dependent inflammatory responses (Luscinskas and Lawler, 1994; Malik and Lo, 1996). Preformed Mac-1 is stored in three separate PMN compartments: secretory vesicles, specific granules, and gelatinase granules (Sengelov, 1996; Borregaard and Cowland, 1997). As such, Mac-1 can be rapidly mobilized to the PMN surface after exposure to degranulation stimuli such as the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP), as well as to weaker stimuli that mobilize only the secretory vesicles (Altieri and Edgington, 1988). In human PMNs, these latter stimuli include LPS and TNF- $\alpha$  among others. Inflammatory stimuli can also promote transcription and translation of Mac-1 genes, thus prolonging integrin involvement during inflammation.

Even when Mac-1 is incorporated into the plasma membrane of activated cells, only a small percentage (~10%) may be competent for ligand binding (Diamond and Springer, 1993). In addition, small numbers of inactive Mac-1 molecules, incapable of binding ligand, are constitutively present on the PMN. Thus, physical appearance of integrins in the cell membrane does not necessarily translate into functional up-regulation for competent ligand binding. The ligand binding site on membrane Mac-1 lies within an extracellular region of

the  $\alpha$ -subunit called "inserted domain" (I domain) that is inaccessible to potential ligands unless integrins are activated by intracellular or extracellular signals (Stanley and Hogg, 1998) (Fig. 2). Near the I domain is a divalent metal binding motif called the "metal ion-dependent adhesion site", or MIDAS. When bound by divalent ions such as manganese or other divalent metals (excluding calcium), the I domain undergoes a conformational change that exposes a requisite epitope for ligand binding (Michishita et al., 1993; Oxvig and Springer, 1999). Another important domain on the  $\text{NH}_2$  terminus of the  $\alpha$ -subunit is a series of seven repeated sequences that form a  $\beta$ -propeller domain and possess putative calcium binding sites that modify binding affinity to ligands (Springer, 1997; Oxvig and Springer, 1998). Integrin activation involves a conformational change such that sites on both the  $\beta$ -propeller and I domains are exposed for ligand binding. An I-like domain also exists on the  $\beta$ -subunit and is necessary for binding to some ligands.

Sites on the intracellular portion of the CD18 subunit are critical for internalization and down-regulation of bound Mac-1 (Rabb et al., 1993). Deletion studies have shown a prolongation of Mac-1 binding to extracellular ligands when critical cytoplasmic residues are missing. In addition, the cytoplasmic tail of CD18 of Mac-1 possesses serine and threonine sites for potential modification by phosphorylation. Granular Mac-1 is not phosphorylated, but it becomes phosphorylated shortly after mobilization and incorporation into the plasma membrane (Buyon et al., 1997). Phosphorylation of CD18 is stimulus-dependent and, although associated with activation, whether it causes an increase in the avidity for a ligand is unclear (Buyon et al., 1997; Valmu et al., 1999).

L-plastin is an actin-bundling protein present in PMNs, phosphorylation of which by phosphoinositide 3-kinase induces CD18-dependent adhesion (Jones et al., 1998b). Bundling of actin might promote the lateral

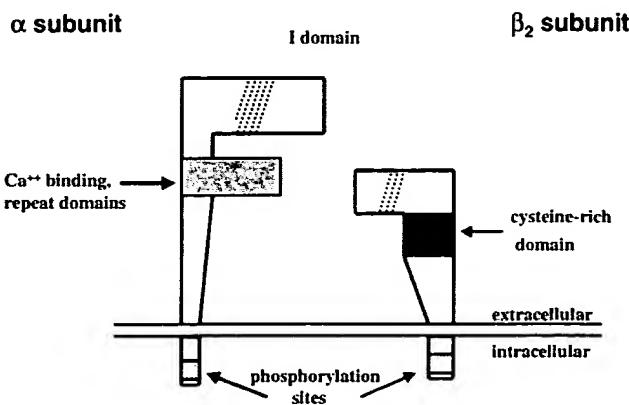


FIG. 2. Schematic representation of the integrin heterodimer. Sites involved in ligand recognition on the  $\alpha$ -subunit include the I domain and  $\text{Ca}^{2+}$ -binding repeat domains. An I-like domain on  $\beta$ -subunits is also involved in ligand recognition. Intracellular phosphorylation sites modulate binding affinity.

mobility of integrins within the cell membrane and facilitate their localization with opposing ligands on the vessel wall. Other studies have elucidated a role for a novel and partially characterized intracellular lipid (Hermanowski-Vosatka et al., 1992; Detmers et al., 1994; Klugewitz et al., 1997). The lipid, integrin-modulating factor-1 is produced by human PMNs on exposure to LPS or to the PMN chemoattractant, interleukin (IL)-8. Production of integrin-modulating factor-1 significantly augments CD18-ligand binding in both intact membranes and in a cell-free system that employs a soluble form of Mac-1. Another intercellular mediator known to potentiate CD18 binding is the protein, cytohesin-1. Although not confirmed to interact with Mac-1, the protein does interact with CD18 in LFA-1 and enhances binding to endothelial ligands (Kolanus et al., 1996).

Several studies suggest that a common intracellular pathway for integrin up-regulation may be via the activation of the G protein Rho. Rho is a small GTPase that is down-regulated by cAMP-dependent protein kinase A. Activators of PMN integrins such as TNF- $\alpha$  and IL-8 decrease intracellular cAMP and thus release inhibition of Rho (Laudanna et al., 1996, 1997). Furthermore, cAMP analogs or phosphatase inhibitors that prolong the elevation of intracellular cAMP inhibit activation of integrins.

In addition to its endothelial ligands, Mac-1 has specific recognition and binding sites for fibrinogen, LPS, factor X coagulation protein, complement protein C3i, heparin, and glycosaminoglycan (Wright et al., 1988; Ross and Vetvicka, 1993; Flaherty et al., 1997). Thus, during some inflammatory scenarios, many ligands for Mac-1 may be present at the same time. For example, during endotoxemia, activation of the complement cascade results in generation of C3i proteins and increased expression of endothelial ligands. Fibrinogen and factor X are normally present in blood. Therefore, CD18-mediated PMN functions might become dysregulated during endotoxemia and thereby affect normal migration processes (reviewed in Wagner and Roth, 1999).

**2. Intercellular Adhesion Molecules.** An important complementary endothelial ligand for Mac-1 is intercellular adhesion molecule-1 (ICAM-1) (CD54), an Ig-like molecule that exhibits low constitutive presentation on EC membranes but is markedly induced by exposure of ECs to inflammatory cytokines (Gerritsen and Bloor, 1993; Hashimoto et al., 1994; Klein et al., 1995; Scholz et al., 1996; Iigo et al., 1997). ICAM-1 is also found on tissue epithelial cells including pneumocytes (Barton et al., 1995; Burke-Gaffney and Hellewell, 1996). LFA-1 can bind to ICAM-1, but it has higher affinity to a related protein, ICAM-2, a ligand to which Mac-1 binds with less affinity. Recent in vitro studies with human umbilical vein ECs demonstrate Mac-1-mediated adhesion and TEM that is partially independent of both ICAM-1 and ICAM-2 (Issekutz et al., 1999).

Vascular cell adhesion molecule-1 (VCAM-1) is also an Ig-like molecule on ECs, but it binds selectively to  $\beta_1$ -instead of  $\beta_2$ -integrins. VCAM-1 interaction with  $\beta_1$ -integrins is critical to migration of monocytes and eosinophils. Recently however,  $\alpha_4\beta_1$ -integrin [very late antigen-4 (VLA-4)] has been identified on both activated human and rat PMNs and can mediate VCAM-1-dependent adhesion of PMNs to endothelium in vitro (Reinhardt et al., 1997; Davenpeck et al., 1998a). Prolonged engagement of endothelial ICAM-1 with cross-linking antibodies or integrin ligands causes the expression of both ICAM-1 and VCAM-1 on ECs (Clayton et al., 1998). Thus, PMN migration in vivo might involve both ICAM-1 and VCAM-1 adhesive pathways.

#### *C. Polymorphonuclear Leukocyte Homing to Transmigration Sites on Endothelium*

Egress of PMNs through EC monolayers occurs preferentially at tricellular junctions (Burns et al., 1997a). Although the mechanism of extravasation is imperfectly understood, it is clear that selectin involvement has been down-regulated at this time and an IgG-type adhesion molecule, platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31), becomes critical for the actual passage of PMNs between ECs (Vaporciyan et al., 1993; Dejana et al., 1995; Newman, 1997).

Found on PMNs, platelets, and ECs, PECAM-1 can serve as its own ligand and form homodimers with molecules on opposing cells. PECAM-1 is evenly distributed over the surface of circulating PMNs and is concentrated at intercellular junctions of unstimulated ECs (Newman, 1997). By virtue of its junctional location, PECAM-1 is hypothesized to be a homing receptor to locate the transendothelial portal for the migrating PMN. Treatment of PMNs or endothelial monolayers with an antibody to PECAM-1 blocks transmigration in vitro (Muller et al., 1993; Muller, 1995), and similar antibodies have been effective in inhibiting PMN migration in rat models of peritonitis and alveolitis (Vaporciyan et al., 1993; Bogen et al., 1994; Muller, 1995). In both whole animal and cell systems, PECAM-1 antibody treatment does not block adhesion.

#### *D. Transmigration*

Compared with the aforementioned processes of margination and adhesion, there is relatively little known of the mechanisms of PMN egress and migration through the subendothelial matrix. PMNs possess proteases capable of digesting collagen, laminin, and other extracellular components present in the vascular wall. Adhesion and migration is accompanied by release of PMN-derived proteases (Wright and Gallin, 1979; Hanlon et al., 1991), and both chemotaxis and migration through artificial substrates in vitro is inhibited by treatment with antiproteases (Lomas et al., 1995; Delclaux et al., 1996). Hence, a critical role has been proposed for PMN-derived protease activity during extravascular transit.

However, protease inhibitors are ineffective in stopping PMN migration through intact EC monolayers and basement membrane matrices *in vitro* (Allport et al., 1997a; Mackeral et al., 1999). Furthermore, mice deficient in gelatinase B (a PMN-derived collagenase) have normal PMN emigration into the lungs, peritoneum, and skin (Betsuyaku et al., 1999). Thus, requirements for protease release and digestion of extravascular matrix components during adhesion and migration are not certain.

Leukocytes bind to matrix components such as collagen, vitronectin, and laminin via  $\beta_1$ -integrins. As mentioned above, activated PMNs can express the  $\beta_1$ -integrin VLA-4, and this allows for their binding to endothelial VCAM-1 *in vitro*. Interaction with  $\beta_1$ -integrins might also be important for transit of PMNs through the extravascular milieu. For example, PMN migration through lung or synovial fibroblast barriers has recently been shown to require not only CD18 ( $\beta_2$ ), but also VLA-4, VLA-5 ( $\alpha_5\beta_1$ ), VLA-6 ( $\alpha_6\beta_1$ ), and VLA-9 ( $\alpha_9\beta_1$ ) (Shang and Issekutz, 1997; Shang et al., 1999). VLA-5 and VLA-6 bind fibronectin and laminin, respectively. Like VLA-4, VLA-9 interacts with fibronectin and VCAM-1, but it also binds to the matrix protein tenascin. Furthermore, VLA-9 is the most highly expressed  $\beta_1$ -integrin on PMNs and is not expressed by either lymphocytes or monocytes (Shang et al., 1999). Fibroblasts and parenchymal tissues such as pulmonary epithelium and hepatocytes express both VCAM-1 and ICAM-1. As such, VLA-9 and Mac-1 appear to be the more critical integrins responsible for PMN migration through the subendothelium.

Lastly, there is evidence for the involvement of PECAM in extravascular transit of leukocytes. Unlike the homotypic interactions between PECAM molecules that mediate homing and diapedesis, migration through the subendothelial environment requires heterophilic binding of leukocytic PECAM-1 to an unidentified ligand (Liao et al., 1995; Wakelin et al., 1996). Antibodies to an amino-terminal domain of PECAM-1 inhibit homophilic binding, whereas antibodies to a membrane-proximal extracellular domain of PECAM blocks heterophilic interactions and movement across the basement membrane (reviewed in Muller and Randolph, 1999).

#### *E. Migration as an Ordered Sequence of Events*

Multiple lines of evidence suggest that selectin activation and binding is required before firm adhesion can occur via Mac-1-ICAM-1 interactions (Lawrence and Springer, 1991; von Andrian et al., 1992; Ley et al., 1995). As mentioned previously, selectin-mediated signals are incorporated with other extracellular inflammatory stimuli to regulate the timely expression and engagement of PMN integrins (Crockett-Torabi and Fantone, 1995; Crockett-Torabi, 1998; Williams and Solomkin, 1999). For example, studies *in vitro* demonstrate that cross-linking activation of L-selectin on PMNs can lead directly to  $\beta_2$ -integrin-mediated adhe-

sion in both static assays (Simon et al., 1995, 1999; Steeber et al., 1997) and in shear-flow models (Gopalan et al., 1997). Furthermore, removal of L-selectin from PMNs renders them incapable of integrin-mediated firm adhesion to endothelial monolayers (Endemann et al., 1997; Zouki et al., 1997). These results are not surprising for flow systems; L-selectin binds more rapidly and at higher shear rates than CD18 integrins (Taylor et al., 1996). However, the requirement for L-selectin in static systems suggests that selectin-binding is required for more than just physical capture of PMNs. Stimulation of leukocytes via L-selectin can activate G-proteins, tyrosine kinases, release of ceramide, and assembly of filamentous actin (Brenner et al., 1997, 1998; Simon et al., 1999), events that may be a required prelude to integrin-dependent adhesion. Firm adhesion by L-selectin activation is blocked by inhibitors of protein tyrosine kinase and protein kinase C, suggesting that these pathways link L-selectin to Mac-1 up-regulation (Steeber et al., 1997).

Engagement of PSGL-1 can modulate Mac-1 function. Binding of PSGL-1 to P-selectin activates tyrosine kinase and leads to the up-regulation and avidity of CD18 on PMNs (Hidari et al., 1997; Blanks et al., 1998). In addition, integrin-activating chemoattractants cause a redistribution of PSGL-1 on PMNs and concomitant loss of P-selectin binding (Lorant et al., 1995). Thus, signal transduction pathways after selectin engagement appear to promote "bond-trading" from selectins to integrins by down-regulating the former and inducing functional expression of the latter.

Activation of CD18 integrins, in turn, can lead to phosphorylation of tyrosine residues on PECAM-1 and potentially modulate the function of these adhesive proteins during PMN transmigration (Lu et al., 1996). Conversely, activation of PECAM-1 on PMNs with either cross-linking antibodies or antigen-binding fragments can increase the activity of Mac-1 (Berman and Muller, 1995), suggesting that cross-talk between the molecules occurs during TEM. Because the trailing end of the migrating PMN must detach from ECs as the leading uropod attaches, the coordination of both Mac-1-ICAM-1 and PECAM-PECAM interactions is critical for successful diapedesis. Engagement of PECAM up-regulates CD18 through phosphoinositide 3-kinase, which is a different pathway for CD18 activation from G-protein-associated activators such as fMLP, IL-8, and C5a (Jones et al., 1998a; Pellegatta et al., 1998). These studies suggest that activation of PMN adhesion molecules via integrin-ligand or selectin-ligand binding may differ from pathways initiated by cytokine-receptor binding.

Binding of Mac-1 to ICAM-1 can cause structural changes in endothelial cytoskeletal proteins associated with adherens junctions without causing EC retraction or injury to monolayers (Del Maschio et al., 1996; Allport et al., 1997b). The endothelial junctional proteins plakoglobin, cadherin, and  $\alpha$ - and  $\beta$ -catenin dissociate within

minutes of PMN binding to endothelium (Del Maschio et al., 1996). It is unclear if this reorganization is required for TEM. After prolonged exposure of endothelial monolayers to inflammatory cytokines, PECAM-1 becomes diffusely distributed throughout the cell membrane and away from intercellular junctions (Romer et al., 1995), whereas L-selectin ligands and ICAM-1 redistribute from random expression to localization at cellular junctions (Bradley and Pober, 1996). It is unknown what role this plays in PMN emigration; in each system, neither ICAM-1, PECAM-1, nor the L-selectin ligand was bound by PMN counterparts. Taken together, control of TEM is dictated in part by intracellular signaling and cross-talk between adhesive proteins and may be separate from pathways elicited by cytokines and chemoattractants that require receptor activation.

#### F. Soluble Mediators of Migration

Promigratory stimuli can be classified generally as either nonchemotactic cytokines or chemoattractants. Canonical inflammatory cytokines, such as TNF- $\alpha$  and IL-1 can engender expression of adhesive proteins on PMNs and ECs, but they are not by themselves chemotactic for PMNs. For example, TNF- $\alpha$  and IL-1, along with other inflammatory mediators, promote the firm adhesion of PMNs to endothelium in systems *in vitro* (Schleimer and Rutledge, 1986; Huber et al., 1991; Burke-Gaffney and Hellewell, 1996; Komatsu et al., 1997). However, egress and migration of PMNs into extravascular spaces requires the presence of chemoattractants that cause the directed migration of PMNs through tissue. Some chemoattractants can promote expression of adhesion molecules on PMNs similar to the responses elicited by IL-1 and TNF- $\alpha$ .

1. *Cytokines: Tumor Necrosis Factor- $\alpha$  and Interleukin-1.* Two of the most important pro-adhesive cytokines that are present during most inflammatory responses are TNF- $\alpha$  and IL-1 (Table 1). The macrophage/monocyte is the primary cellular source of both TNF- $\alpha$  and IL-1, and LPS is perhaps their most important inducer (reviewed in Tracey and Cerami, 1993, 1994; Bemelmans et al., 1996; Di Girolamo et al., 1997). Among many pathophysiologic effects of TNF- $\alpha$  are shock, cytotoxicity, and cachexia. Binding to two different TNF- $\alpha$  receptors present on each cell induces the effects of TNF- $\alpha$  on PMNs and ECs. PMNs can respond to TNF- $\alpha$  by activating and expressing integrins, producing platelet-activating factor (PAF) and other mediators, and releasing granule contents. Likewise, ECs mobilize selectins, up-regulate ICAM-1, and activate procoagulant pathways in response to TNF- $\alpha$  exposure. During inflammation and endotoxemia, PMNs release from their membranes a soluble TNF- $\alpha$  receptor that can bind to and effectively inactivate circulating TNF- $\alpha$ .

IL-1 has been known under various names for more than 40 years as an important mediator of inflammation and fever (reviewed in Kampschmidt, 1984; Movat,

TABLE 1  
Selected mediators of PMN migration and their effects

	Summary of Effects
Cytokines	
TNF	Induces P-selectin, ICAM-1 and IL-8 in ECs, shedding of L-selectin, expression of CD18 on PMNs, inhibition of PMN chemotaxis
IL-1	Induces P-selectin, ICAM-1 in ECs, shedding of L-selectin, expression of CD18 on PMNs
IFN- $\gamma$	Induces P-selectin, ICAM-1 in ECs, shedding of L-selectin, expression of CD18 on PMNs
Chemoattractants	
PAF	PMN chemotaxin, induces EC and PMN expression of adhesion molecules
LTB <sub>4</sub>	PMN chemotaxin, induces EC and PMN expression of adhesion molecules
FMLP	PMN chemotaxin, induces EC and PMN expression of adhesion molecules, desensitizes CXC receptors
C5a	PMN chemotaxin, induces PMN adhesion molecules, desensitizes PMNs to other chemoattractants
Chemokines <sup>a</sup> (CXC)	
IL-8	Chemotactic for human, rabbit, and bovine PMNs; induces shedding of L-selectin and expression of CD18 on PMNs; systemic administration inhibits PMN migration <i>in vivo</i>
MIP-2 (CINC-3)	Chemotactic for rat PMNs, induces shedding of L-selectin and expression of CD18 on PMNs

IFN- $\gamma$ , interferon- $\gamma$ .

<sup>a</sup> See Rollins (1997) for a detailed review on chemokines.

1987; Le and Vilcek, 1987; Moldawer, 1994). Its cellular sources and physiologic effects are similar to those of TNF- $\alpha$ , and the two are often found together in a variety of inflammatory scenarios. Like TNF- $\alpha$ , IL-1 induces selectin and ICAM-1 expression on ECs and promotes integrin activation on PMNs. Exposure of EC monolayers or PMNs *in vitro* to TNF- $\alpha$  (Romer et al., 1995; Bradley and Pober, 1996; Burke-Gaffney and Hellewell, 1996) or IL-1 (Schleimer and Rutledge, 1986; Scholz et al., 1996) causes time- and dose-dependent expression of selectins and integrins. In addition, TNF- $\alpha$  and IL-1 treatment *in vivo* induces ICAM-1 in lung and small intestine (Komatsu et al., 1997). Thus, the early appearance of TNF- $\alpha$  and IL-1 in plasma during inflammation is likely critical for the capture and firm adhesion of PMNs to vascular endothelium. During inflammation, PMNs and macrophages express on their membranes a receptor antagonist (IL-1ra) that binds to IL-1, but it is not linked to signal transduction machinery. A similar decoy receptor has not been identified in ECs. Such receptors likely serve to regulate the magnitude of inflammation.

Although LPS, TNF- $\alpha$ , and IL-1 are not themselves chemotactic for PMNs, their exposure to ECs can elicit TEM *in vitro*. This phenomenon is dependent on cytokine-stimulated production of endothelial-derived chemoattractants that can be detected in the culture medium during migration assays *in vitro* (Huber et al., 1991; Kuijpers et al., 1992; Smart and Casale, 1994; Burns et al., 1997b).

2. *Chemoattractants.* PMNs have at least five different receptors for chemotactic stimuli. Unique receptors

exist for PAF, complement protein C5a, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and bacterial peptides (e.g., fMLP) (Table 1). In addition, a growing number of chemokines and their specific receptors are being discovered and characterized as important players in inflammatory responses of PMNs (Furie and Randolph, 1995). Chemoattractants/chemokines for PMNs can be produced by a wide variety of cells, including endothelial and epithelial cells, macrophages, monocytes, lymphocytes, platelets, PMNs, and parenchymal cells. In models *in vitro*, chemoattractants can activate PMNs or ECs to express adhesive proteins in a manner similar to TNF- $\alpha$  or IL-1. Thus, redundant pathways for adhesive and migratory processes probably occur *in vivo* (Detmers et al., 1990, 1991; Huber et al., 1991).

a. *Platelet-activating factor.* PAF is an acetylated phosphoglyceride derived from lipids of cell membranes and is produced by ECs, platelets, PMNs, and macrophages. It promotes both pro-inflammatory and pro-adhesive processes. During systemic sepsis and bacterial pneumonia, PAF is required for maximal PMN responses (Makristathis et al., 1993; Mathiak et al., 1997). Furthermore, infusion of PAF into animals can reproduce some of the inflammatory responses to endotoxemia. In addition to chemotaxis, PAF may be important in mediating firm adhesion. For example, EC-derived PAF can be localized on the EC surface where it has access to PAF receptors on rolling PMNs (Zimmerman et al., 1996b).

b. *Leukotriene B<sub>4</sub>.* LTB<sub>4</sub> is produced by monocytes and PMNs from arachidonic acid (reviewed in McMillan and Foster, 1988; Borgeat and Naccache, 1990; Brooks and Summers, 1996). It is present in most inflammatory foci where it is 10 to 1000 times more potent than PAF at eliciting chemotactic responses of PMNs. In addition, LTB<sub>4</sub> can elicit PMN adherence to ECs and to artificial surfaces. Adherence to endothelium is due in part to the direct action of LTB<sub>4</sub> on the EC; however, an endothelial receptor for LTB<sub>4</sub> has not been identified (Nohgawa et al., 1997).

c. *Complement protein C5a.* Cleavage of complement protein C5 yields C5b, a component of the membrane attack complex, and C5a, a powerful chemoattractant. Circulating C5a is produced during the activation of the classical complement cascade in blood and, once formed, can bind immediately to circulating PMNs (Kohl and Bitter-Suermann, 1993). Proteins of the alternative complement pathway, which includes C5, can be produced by tissue macrophages and specialized epithelial cells, including type II pneumocytes (Strunk et al., 1988). As such, extravascular C5a promotes gradient-dependent TEM of PMNs. In addition, engagement of the PMN receptor for C5a can promote activation of secretory and oxidase pathways.

d. *Formyl-methionyl-leucyl-phenylalanine.* Formylated bacterial peptides result from the cleavage of the NH<sub>2</sub>-terminal portions of common bacterial proteins

during synthesis (Thelen et al., 1993). Similar formylated peptides are not found in mammalian cells. fMLP receptors are expressed on unstimulated PMNs, but activation with fMLP or other agents can cause the mobilization of secretory vesicles in which 2- to 5-fold more receptors are sequestered (Borregaard and Cowland, 1997). Like C5a, fMLP exerts pluripotent activities, including degranulation, oxidative burst, cytoskeletal changes, chemotaxis, and priming for enhanced response to other activators.

e. *Chemokines.* Chemokines are a group of approximately 40 small proteins (6 to 15 kD) with similar, cysteinyl-containing structures (reviewed in Rollins, 1997; Nickel et al., 1999). The most well studied PMN chemokine is IL-8, which is the primary stimulus for PMN migration in many inflammatory responses in humans and rabbits (Table 1). Chemokines were first identified *in vitro* and initially thought to be produced only by activated macrophages and monocytes. However, under the proper conditions, their production and release has been elicited from neutrophils, endothelium, epithelium, platelets, and a variety of parenchymal cells *in vitro* (Huang et al., 1992; Xing et al., 1994; Crippen et al., 1995; Furie and Randolph, 1995). Further studies suggest that these cells produce chemokines in animal models (Rovai et al., 1998).

Structurally, all chemokines possess four cysteine residues in their amino-terminal end that form disulfide bridges. Chemokines are classified by the sequence of the two most NH<sub>2</sub>-proximal cysteines. In  $\alpha$ - or CXC chemokines, the cysteines are separated by an amino acid (X). In  $\beta$ - or CC chemokines, the cysteines are adjacent to one another. The structural difference is related to their ability to elicit distinct leukocyte migration. In general, PMN responses are invoked by  $\alpha$ -chemokines, and mononuclear cells respond most strongly to  $\beta$ -chemokines.

In addition to IL-8, at least six other CXC chemokines mediate PMN responses in humans: neutrophil-activating peptide-2, three forms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of growth-related oncogenes/melanoma growth-stimulating activity, and epithelial cell-derived neutrophil-activating peptide-78. Different roles for two PMN receptors, CXCR1 and CXCR2, have been characterized. IL-8 can bind to either receptor, but the remaining six proteins appear to have affinity for only CXCR2.

Rodents do not have an IL-8 analog and instead possess cytokine-induced neutrophil chemoattractants (CINCs) that are similar to growth-related oncogene proteins (Watanabe et al., 1993; Nakagawa et al., 1994). Two in particular, CINC, also known as KC or CINC-1, and macrophage inflammatory protein-2 (MIP-2), which has been referred to as CINC-3, are critical to inflammatory responses in mice and rats. Recently, two more CINC-related proteins have been identified and a nomenclature as CINC-2 $\alpha$  and CINC-2 $\beta$  proposed. All four are released from LPS-stimulated rat macrophages in

vitro and possess similar abilities to elicit chemotaxis and degranulation of PMNs (Shibata et al., 1995; Nakagawa et al., 1996). Lastly, a CXC chemokine elicited from murine fibroblasts, LPS-induced CXC chemokine (LIX), has been identified in isolated cells and in endotoxemic animals (Smith and Herschman, 1995; Rovai et al., 1998). Results from binding studies with rodent  $\alpha$ -chemokines and PMNs show a high-affinity receptor for MIP-2 and a shared receptor for the other three CINC (Murakami et al., 1997; Zagorski and Wahl, 1997). Receptor interactions by LIX have not been described in detail.

Critical roles for rodent CXC chemokines have been demonstrated in various rat models of inflammation. Both CINC and MIP-2 are involved in pulmonary PMN responses during bacterial pneumonia, ozone and silica dust inhalation, and immune-complex deposition (Huang et al., 1992; Driscoll et al., 1993, 1996; Frevert et al., 1995a,b; Koto et al., 1997; Shanley et al., 1997). Secretion of MIP-2 has been demonstrated in epithelial cells of the small intestine (Ohno et al., 1997b) and tubulointerstitial cells from kidney (Tang et al., 1997) and has been implicated in models of arthritis (Schimmer et al., 1997), allergic inflammation (Xiao et al., 1997), and liver injury (Jaeschke, 1996).

**3. Cytokine-Chemoattractant Interaction during Migration.** Although LPS, TNF- $\alpha$ , and IL-1 are not chemotactic for PMNs, their exposure to cultured cells can induce the production of chemoattractants such as IL-8 and PAF (Huber et al., 1991; Kuijpers et al., 1992; Smart and Casale, 1994; Burns et al., 1997b). For example, IL-8 released into culture medium after TNF- $\alpha$  stimulation of ECs can cause CD18 up-regulation on PMNs (Huber et al., 1991). In addition, EC-derived IL-8 can become localized on the cell surface where it can activate PMNs close to the vessel wall (Kuijpers et al., 1992; Rot, 1993; Imaizumi et al., 1997). Indeed, treatment of EC monolayers with antibody to IL-8 inhibits rolling PMNs from firmly adhering to the monolayer (Rainger et al., 1997), suggesting that localized IL-8 is required for adhesion. The physical nature of the IL-8-EC relationship is unclear. Rot and coworkers (Rot et al., 1996) described *in situ* binding of radiolabeled IL-8 to vascular endothelium in lungs, liver, and kidney. However, there is no evidence for an IL-8 receptor or binding protein on cultured or primary ECs *in vitro* (Petzelbauer et al., 1995; Rot et al., 1996). IL-8 likely binds to components of the vessel wall matrix such as heparan and glycosaminoglycans (Hoogewerf et al., 1997). Thus, localization might not be due to an EC component but to extracellular sites near the cell.

Stimulation of ECs with IL-1 causes IL-8 synthesis and secretion within 1 to 2 h, whereas longer exposures promote the storage of newly synthesized IL-8 in Weibel-Palade bodies (Rot et al., 1996). Furthermore, release of IL-8 from Weibel-Palade bodies could be induced by treatment of cells with phorbol ester or histamine. This

observation raises the possibility that comobilization of endothelial IL-8 with P-selectin might modulate PMN adhesion during chronic inflammation.

These studies suggest a mechanism for the spatial and temporal orderliness of PMN-EC interaction in the face of multiple and conflicting inputs from inflammatory mediators. Cytokines and chemoattractants may promote redundant and perhaps dysregulated signals for firm adhesion when both types of mediators are present (Detmers et al., 1990, 1991; Huber et al., 1991). Untimely expression or activation of adhesive proteins on circulating PMNs might be detrimental to the step-wise dependence of EC-mediated migration processes. This is suggested by results from systems *in vitro* in which pretreatment or cotreatment of PMNs with IL-8, fMLP, LTB<sub>4</sub>, or C5a causes inhibition of adhesion and/or migration across endothelial monolayers (Luscinskas et al., 1992; Moser et al., 1993; Takahashi et al., 1995). Although the effect on integrin expression was not examined, there was a positive correlation between L-selectin shedding and inhibition of migration by the various chemokines (Moser et al., 1993). Similarly, i.v. administration of IL-8 inhibits PMN migration to extravascular sites of inflammation in rabbits (Hechtman et al., 1991; Ley et al., 1993). Similar experiments with rat CINCs have not been performed. However, exposure of isolated rat blood to MIP-2 can cause PMNs to shed L-selectin and increase surface expression of CD11b/CD18 (Frevert et al., 1995b). These are the same responses seen in human PMNs after exposure to IL-8, TNF- $\alpha$ , IL-1, and C5a.

Modulation of TEM by cross-talk among receptors for cytokines, chemokines, chemoattractants, and LPS is depicted in Fig. 3. Although the mechanisms of chemoattractant-induced depression of migration are not entirely clear, results from studies *in vitro* suggest that receptor desensitization may be involved (Campbell et al., 1997). For example, C5a-treated PMNs demonstrate decreased chemotactic responses (Kitayama et al., 1997), and such treatment can induce cross-desensitization of IL-8 and fMLP receptors in systems *in vitro* (Tomhave et al., 1994; Blackwood et al., 1996; Sabroe et al., 1997). Likewise, treatment of neutrophils with fMLP or C5a down-regulates IL-8 receptors (Campbell et al., 1997; Richardson et al., 1998). In addition, TNF- $\alpha$  can inhibit PMN migration in models *ex vivo* and *in vivo* (Otsuka et al., 1990), presumably because the PMN receptor for TNF- $\alpha$  is functionally linked to chemotactic receptors (Schleiffenbaum and Fehr, 1990; Balazovich et al., 1996). Both TNF- $\alpha$  and LPS down-modulate CXC receptors by a metalloproteinase-mediated mechanism (Khandaker et al., 1999). Inhibitor studies suggest that the metalloprotease is distinct from the enzyme responsible for cleavage of L-selectin after exposure to cytokines and chemoattractants (Kishimoto et al., 1995; Sadallah et al., 1999).

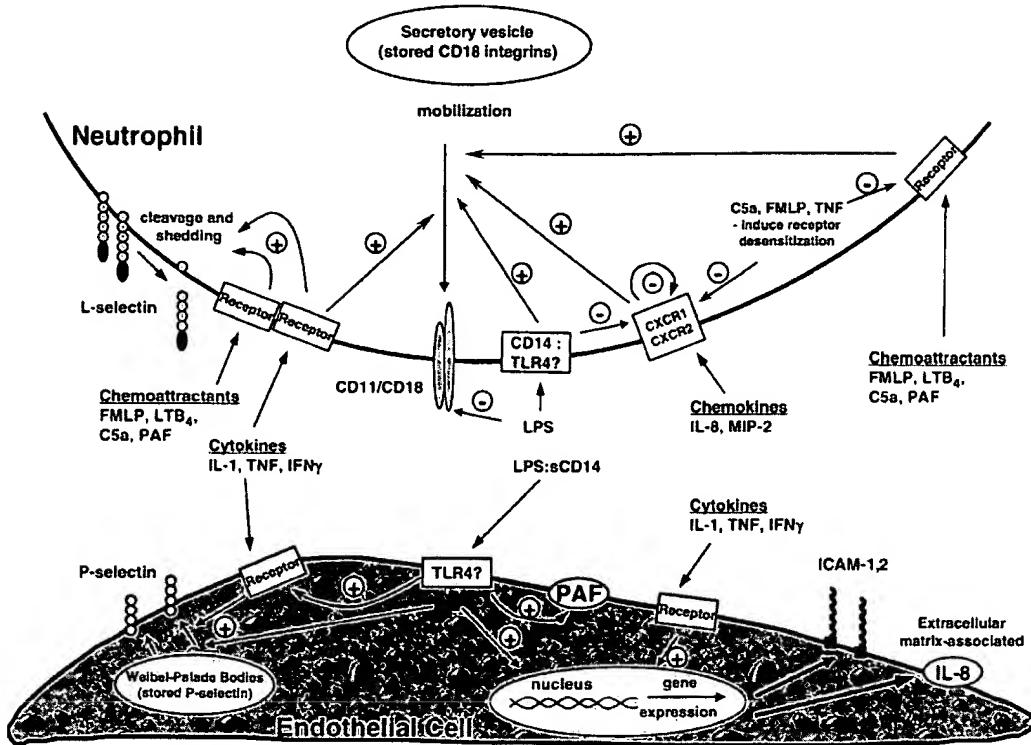


FIG. 3. Schematic representation of the modulation of adhesive interactions by LPS, cytokines, chemokines, and other chemoattractants. Numerous mediators can cause PMNs to shed L-selectin and promote the mobilization of CD11/CD18 to the PMN membrane in the transition from vascular rolling to adherence. Chemokines down-regulate their receptors by phosphorylation and internalization, whereas LPS and TNF- $\alpha$  indirectly cause proteolytic cleavage of these receptors. C5a, fMLP, and TNF- $\alpha$  down-regulate both chemokine and certain other chemoattractant receptors as described in the text. LPS can bind to CD11/CD18 and may interfere with ligand binding and activate intracellular pathways. In ECs, LPS and cytokines cause the mobilization of Weibel-Palade bodies to the cell membrane and induce gene expression and synthesis of ICAMs and IL-8. LPS also causes the production and membrane localization of PAF. TLR-4, Toll-like receptor 4.

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Bacterial endotoxin is probably the most potent known inflammanogen and can directly and indirectly modulates PMN migration. The presence of LPS in the blood (endotoxemia) is often associated with circulating TNF- $\alpha$ , IL-1, IL-8, C5a, and other soluble mediators that are capable of modulating PMN function (Wagner and Roth, 1999). Although LPS can induce the expression of CD18 on PMNs, it can also down-regulate CXCR1 and CXCR2 and inhibit CD18-mediated chemotaxis in vitro (Bignold et al., 1991; Khandaker et al., 1998). Furthermore, LPS binds to CD18 where it may interfere with ICAM binding (Flaherty et al., 1997). Although it is appreciated that LPS binds to the glycosylphosphatidylinositol-linked membrane protein CD14, several studies demonstrate that a co-receptor, namely Toll-like receptor 4, transduces intracellular signaling by LPS in inflammatory cells (Chow et al., 1999; Lien et al., 2000). A similar role for CD18 as a signaling partner for CD14 has been postulated (Flaherty et al., 1997; Todd and Petty, 1997). PMNs isolated from endotoxemic subjects and PMNs exposed in vitro to LPS have dysfunctional adhesive and migratory responses that may be related to altered CD18 signaling (Wagner and Roth, 1999).

#### G. Implications

TEM of PMNs is a carefully orchestrated sequence of cell activation, adhesion molecule expression, and molecular cross-talk between receptors on both cell types. As such, there are several opportunities to modulate and modify the migratory response by both exogenous and endogenous factors. Several lines of evidence suggest that integrin activation needs to occur at the PMN-EC interface and not in the circulation. Premature activation of PMN integrins and chemotactic receptors by circulating mediators in vivo might be avoided by temporally and spatially controlled release and concentration of chemokines at the endothelium-blood interface as suggested by Rot and coworkers (Rot, 1993; Rot et al., 1996). Knowledge of the molecular basis of control of TEM by endogenous factors (i.e., cytokines and chemokines, etc.) may suggest how therapeutic interventions might be formulated to alter undesirable recruitment of inflammatory cells. In this regard, pharmacologic manipulation of adhesion molecules by a variety of agents has shown promise in both human and animal models of inflammation. Several therapeutic approaches are discussed in the last section of this review, but first the

special case of adhesive interactions that occur during pulmonary inflammation is presented in the next section.

### III. Polymorphonuclear Leukocyte Migration in the Lungs

Most of the known molecular and cellular mechanisms of TEM have been elucidated from studies in vitro and in systemic vessels in the mesentery and dermis. Recent work in lung, however, suggests that PMN trafficking in the pulmonary circulation is fundamentally different from events in the systemic vasculature. In animal models tested so far, PMN behavior in lungs differs with respect to 1) the marginated pool of PMNs, 2) the site of TEM, and 3) requirements of adhesion molecules for PMN extravasation (Fig. 4). Although, the basic mechanisms of TEM remain incompletely understood, these three differences suggest that different paradigms are needed to explain the kinetics of PMN migration in different vascular beds.

#### A. Marginated Pool of Polymorphonuclear Leukocytes

Margination of PMNs is a prerequisite for their escape from the main flux of flowing blood, where they can sense and respond to inflammatory signals present in vessel wall microenvironment. The concentration of PMNs within pulmonary capillary blood is 35 to 100 times greater than in large vessels of the systemic circulation (Doerschuk et al., 1987, 1993; Gee and Albertine, 1993). In the systemic circulation, margination occurs as rolling in postcapillary venules and is mediated by L-selectin and P-selectin and their respective ligands (Butcher, 1991; Lawrence and Springer, 1991; Spertini et al., 1991; Tozeren and Ley, 1992). However, 97% of pulmonary vascular PMNs are found in the capillary network where vessels are too small to allow rolling (2–15  $\mu\text{m}$ ) (Doerschuk et al., 1993). Selectin-mediated rolling still occurs in pulmonary venules ( $>15 \mu\text{m}$ ), but it makes a small contribution to the total pool of marginated PMNs in the pulmonary circulation.

Despite the lack of rolling in pulmonary capillaries, selectins may be involved in the PMN-EC interactions.

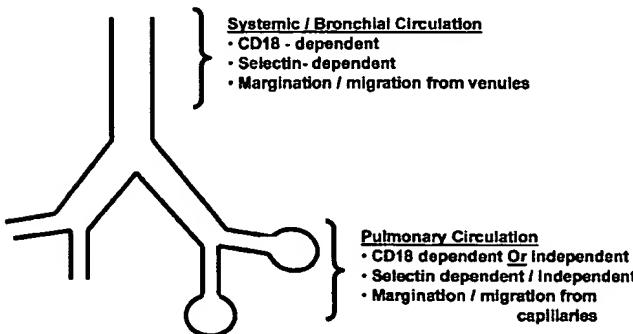


FIG. 4. Differences in PMN migration occurring in pulmonary circulation and in bronchial airway and systemic circulations.

For instance, treating rabbits with fucoidan, a polysaccharide made mostly of sulfated fucose that inhibits binding of all selectins, decreases the frequency of PMN stopping in capillaries by 25% and the duration of stops by 50% (Kuebler et al., 1997). Similar results are seen in fucoidan-treated rats, in which PMN localization in alveolar capillaries is decreased by 15% (Yamaguchi et al., 1997). In the same study in rats, an antibody to P-selectin was ineffective in slowing PMN transit through the lungs, whereas rolling on systemic venules was significantly inhibited. Because E-selectin is not expressed in noninflamed rat lungs, all selectin-mediated binding is therefore due to L-selectin. Furthermore, despite the expression of ICAM-1 in normal rat pulmonary capillaries, treatment with antibodies to ICAM-1 does not affect PMN transit times (Yamaguchi et al., 1997). Thus, during nonpathologic conditions, L-selectin is the only adhesion molecule likely to be involved in pulmonary PMN margination in rat.

Another factor to account for the dramatic extent of pulmonary PMN margination may be the discrepancy between neutrophil and capillary diameters (Downey et al., 1990). PMNs that are 7 to 8  $\mu\text{m}$  in diameter must deform to an elongated shape to pass through capillaries, which average 5 to 6  $\mu\text{m}$  and can be as small as 2  $\mu\text{m}$  (Hogg, 1987; Doerschuk et al., 1993; Wiggs et al., 1994; Gebb et al., 1995). Shape change in PMNs takes much longer than in erythrocytes, and this likely accounts for their longer pulmonary transit time compared with erythrocytes (2.7 versus 1.3 s) (Hogg et al., 1988; Lien et al., 1991).

A dramatic increase in pulmonary margination is accompanied by neutropenia when animals are given i.v. LPS or zymosan-activated serum (ZAS; a source of complement activation products; C5a) (Haslett et al., 1987; Doerschuk, 1992). Capillary sequestration in these models may be due to lack of PMN deformability, inasmuch as these same agents cause actin polymerization and PMN stiffness in vitro (Erzurum et al., 1992).

#### B. Site of Migration

Given their slow transit and intimate contact with capillary endothelium, PMNs are well positioned to respond to inflammatory signals generated within airspaces. Thus, in contrast to migration predominately from postcapillary venules in the systemic circulation, PMNs primarily extravasate from the alveolar capillary network in rabbits, rats, and mice (Doerschuk et al., 1989; Walker et al., 1991; Doerschuk, 1992; Downey et al., 1993). It is curious, therefore, that P-selectin is constitutively expressed on pulmonary arterioles and venules in rabbits and not on pulmonary capillary ECs where margination and extravasation occurs (Mulligan et al., 1992b). In rat, by contrast, P-selectin is absent in the normal lung, but ICAM-1 is constitutively expressed on capillary and venular endothelium (Yamaguchi et al., 1997). As discussed previously, ICAM-1 associates with

CD18 to mediate the firm adhesion of PMNs and usually requires induction for full responses. Although ICAM-1 has a low constitutive level of expression in most tissues, it is 30-fold higher in lung tissue (Panes et al., 1995). Thus, pulmonary leukostasis after infusion of LPS or ZAS may be due to the high level of ICAM-1 in lung (Haslett et al., 1987; Doerschuk et al., 1989; Doerschuk, 1992). Both LPS and ZAS are capable of mobilizing CD18 on isolated PMNs, and similar up-regulation of CD18 in vivo would provide ligands for lung ICAM-1 (Erzurum et al., 1992; Klut et al., 1997). Therefore, by virtue of the density in adhesion molecules and slow PMN transit times, PMN adhesion and extravasation is favored in the pulmonary capillaries over downstream venules.

### C. Adhesion Molecule Requirements

**1. Integrins.** Another important difference between the pulmonary and systemic circulations is in the mechanics of PMN diapedesis, or egress of the PMN through the vessel wall. In all models tested so far, systemic PMN migration from postcapillary venules requires CD18 integrins. However, pulmonary PMN migration can occur independently of CD18 adhesion molecules. Whether or not migration depends on CD18 varies with the intrapulmonary stimulus. IL-1, phorbol myristate acetate, and Gram-negative bacterial stimuli, including LPS, elicit migration via pathways predominately mediated by CD18 (Doerschuk et al., 1990; Hellewell et al., 1994; Qin et al., 1996; Ramamoorthy et al., 1997). By contrast, Gram-positive bacteria, hydrochloric acid, and C5a elicit pulmonary PMN recruitment that is mostly independent of CD18. The adhesion molecules needed for CD18-independent migration in lung are unknown.

Endotoxin derived from Gram-negative bacteria elicits airway PMN migration that requires CD18 and causes the up-regulation of ICAM-1 within pulmonary vessels (Tang et al., 1995; Freeman et al., 1996; Beck-Schimmer et al., 1997). At the same time, expression of L-selectin and CD18 is unchanged on PMNs in the vascular compartment, and only after PMNs arrive into airspaces is L-selectin shed and CD11/CD18 expression increased (Burns and Doerschuk, 1994). By contrast, during CD18-independent migration to Gram-positive organisms, there is no change in ICAM-1, yet CD11/CD18 is up-regulated on vascular PMNs before migration and is expressed to an even greater degree on airway PMNs. Thus, an inverse relationship exists between the requirement for CD18 during PMN migration and CD18 expression on vascular PMNs before migration. As described previously, PMNs can express and adhere via  $\beta_1$ -type integrins to a variety of substrates, including cardiac myocytes, ECs, fibroblasts, fibronectin, and laminin (Reinhardt et al., 1997; Shang and Issekutz, 1997). It remains unresolved if  $\beta_1$ -type integrins mediate CD18-independent migration in vivo. Furthermore,

studies in vitro of CD18-independent TEM have failed to implicate  $\beta_1$ -integrins or any other adhesion molecule examined (Issekutz et al., 1995).

**2. Selectins.** It is unclear if selectins are responsible for CD18-independent migration in vivo. Studies using knockout mice and blocking antibodies that target selectins have not provided definitive answers. Mice deficient in L-, P-, or E-selectins exhibit PMN rolling behavior that is compromised in both normal and inflamed venules in the systemic circulation (Mayadas et al., 1993; Johnson et al., 1995; Kunkel and Ley, 1996). In a model of pneumonia using *Streptococcus pneumonia* organisms (a CD18-independent stimulus), E/P-selectin double-knockout mice had 4 times as many airway PMNs as wild-type mice (Mizgerd et al., 1996), suggesting that selectins suppress CD18-independent migration. However the mutant mice also had basal neutrophilia and margination of pulmonary PMNs that was 8-fold greater than that in wild-type mice. Furthermore, knockout mice also had decreased L-selectin on their PMNs (15% of normal), had increased hematopoietic cytokines, and failed to thrive normally (Bullard et al., 1996; Frenette et al., 1996). Therefore, caution should be used in interpreting results of knockout studies in consideration of compensatory mechanisms that may occur in these animals. These results do, however, suggest that P- and E-selectins are not required for migration of PMN to *S. pneumonia*, a CD18-independent stimulus.

Compared with normal animals, mice deficient in L-selectin are protected against injury and death caused by endotoxemia (Tedder et al., 1995b). The knockout mice had significantly less recruitment of PMNs into the pulmonary vasculature after i.v. LPS. By contrast, pulmonary PMN recruitment in response to *S. pneumonia* was the same in wild-type and L-selectin knockout mice (Doyle et al., 1997). Thus, L-selectin-mediated processes appear to be involved in CD18-dependent migration but not for migration in response to CD18-independent stimuli. Similar results were reported in P- and E-selectin knockout mice. That is, selectins appear to be involved in CD18-dependent migration but not in CD18-independent processes. This is consistent with the sequential selectin-CD18 relationship discussed in the first section of this review.

An alternative method of blocking selectins is with sialyl-oligosaccharides that approximate the structure of natural selectin ligands. Lung injury caused by IgG immune-complex deposition injury is CD18-dependent and is prevented by treating with sialyl compounds (Mulligan et al., 1993). Immune deposition injury is characterized by the formation of immune complexes at the alveolar-capillary interface. Sialyl analogs can also inhibit PMN pulmonary recruitment (by 50%) to airway endotoxin in rats (Hayashi et al., 1999). These results further support the link between selectin- and CD18-mediated pathways.

### D. Models of Pulmonary Polymorphonuclear Leukocyte Migration

PMNs emigrate into pulmonary airspaces of rats in response to a number of different pathogenic and toxic stimuli (Table 2). Although each stimulus induces the production of chemoattractants and other inflammasins, the qualitative and quantitative profile of these mediators can be different for various stimuli. In addition, the adhesion molecule requirement for PMNs varies with the stimulus. Intrapulmonary mediators produced by different stimuli may dictate the requirement (or lack thereof) for CD18 during PMN migration in the lung. Below are described a few of the more well studied airway stimuli that elicit pulmonary PMN emigration.

**1. Bacteria and Bacterial Products.** Rabbits respond to both Gram-positive and Gram-negative pneumonia with airway production of IL-8 and TNF- $\alpha$  (Shoberg et al., 1994). However, IL-8 concentrations in bronchoalveolar lavage fluid are 2-fold greater and TNF- $\alpha$  is 10-fold greater in rabbits with Gram-negative pneumonia compared with animals with Gram-positive pneumonia. The production of CINCs and TNF- $\alpha$  after Gram-negative airway stimuli in rats is well documented and was detailed earlier. It is unknown if Gram-positive stimuli in rat airways elicit a lesser cytokine response than a Gram-positive stimulus, similar to what is observed in the rabbit.

In general, PMN airway accumulation in response to Gram-negative bacterial stimuli requires CD18. The extent of inhibition of PMN migration by CD18 antibodies is 75 to 80% (Doerschuk et al., 1990; Ramamoorthy et al., 1997). Conversely, Gram-positive stimuli elicit PMN emigration that is mostly independent of CD18 pathways, with inhibition by CD18 antibodies ranging from 0 to 45% (Doerschuk et al., 1990; Ramamoorthy et al., 1997). Thus, based on the available data, Gram-positive stimuli appear to be largely CD18-independent, and Gram-negative stimuli are predominately CD18-dependent. Moreover, Gram-negative stimuli appear to elicit greater TNF- $\alpha$  and IL-8 production than do Gram-positive stimuli.

Taken together, the results of CD18 antibody studies and the disparate cytokine production elicited by various stimuli suggest that the adhesion molecule requirement for migration might depend on the type and quantity of cytokines and chemokines produced in response to a stimulus. Further work on this hypothesis is required

to match mediators definitively with adhesion molecule involvement.

**2. Acid Aspiration.** Instillation of hydrochloric acid into the airways of laboratory animals is used to model gastric acid aspiration in humans. Neutrophil accumulation in acid-instilled rabbits is dependent on the generation of airway-derived IL-8 (Folkesson et al., 1995), but it does not require CD18 adhesion molecules (Doerschuk et al., 1990). However, CD18 is required for lung injury in this model. Treatment with antibody to CD18 protects from abnormalities in oxygenation and vascular leak without affecting PMN airway accumulation (Goldman et al., 1995; Folkesson and Matthay, 1997). Acid aspiration can also lead to PMN-mediated injury at sites distal from inoculated lung lobes. For example, PMN accumulation occurs in contralateral lobes but, unlike inoculated lobes, PMN emigration is blocked by CD18 antibodies (Goldman et al., 1995). Thus, direct effects of acid on pulmonary PMN migration seem not to require CD18, but indirect effects (e.g., in contralateral lungs) are CD18-dependent.

Similar responses occur in acid-instilled rats. That is, PMN accumulation in acid-instilled airways is CD18-independent (Motosugi et al., 1998), whereas PMN-mediated injury to remote organs, including heart, kidney, and small intestine, requires CD18 (Goldman et al., 1990; Yamada et al., 1997). In addition, extrapulmonary injury is dependent on complement activation and circulating TNF- $\alpha$ . Blockade of either of these inflammatory mediators prevents remote organ injury and lung vascular leak in the aspirated lobe; however, PMN emigration is unchanged.

In summary, acid instillation induces compartmentalized inflammatory responses. Localized responses include CD18-independent pulmonary PMN emigration and CD18-dependent lung injury. The remote component of injury is mediated by TNF- $\alpha$  and complement products that promote CD18-dependent PMN processes.

**3. Interleukin-1.** Production of IL-1 in rat airways is required for full PMN migratory responses in rat models of immune-complex deposition or inhalation of LPS, quartz dust, or diesel exhaust particles (Kusaka et al., 1990; Ulich et al., 1991; Warren, 1991; Yang et al., 1997). Instillation of IL-1 into rat airways itself is sufficient to induce PMN emigration that is dependent on airway production of the neutrophil chemokines, CINC, and MIP-2 (Xu et al., 1995; Hybertson et al., 1997). Treatments with phospholipase A<sub>2</sub> inhibitors, anti-inflammatory prostaglandins, or inhaled nitric oxide inhibit PMN accumulation after airway instillation of IL-1 without affecting chemokine production (Leff et al., 1994; Guidot et al., 1996; Lee et al., 1997). IL-1 instillation also causes PMN-dependent vascular leak that is linked to oxidant injury (Guidot et al., 1994; Leff et al., 1994).

In rabbits treated intratracheally with IL-1, pulmonary PMN emigration is significantly reduced by anti-

TABLE 2  
Selected animal models of pulmonary PMN migration

Airway Stimulus	CD18 Dependency for Migration
Bacteria and bacterial products	
Gram-negative (LPS; endotoxin)	Dependent
Gram-positive (lipoteichoic acid)	Independent
Acid aspiration (hydrochloric acid)	Independent
IL-1	Dependent
Complement protein C5a	Partial dependency
Immune-complex deposition (IgG)	Partial dependency

bodies to CD18 (Hellewell et al., 1994). Similar studies using CD18 antibodies have not been performed in IL-1-instilled rats.

**4. Complement Protein C5a.** Complement protein C5 is found in the lavage fluid collected from healthy humans and animals. Proteolytic cleavage of C5 during inflammation produces the highly chemotactic fragment, C5a. In rabbits, instillation of C5a into airways causes emigration of PMNs into airspaces that is not significantly affected by antibodies to CD18 (Hellewell et al., 1994).

In studies using C5-deficient mice, PMN emigration during Gram-positive pneumonia did not occur, whereas the PMN migratory response to Gram-negative pneumonia was only delayed by an hour (Larsen et al., 1982; Toews and Vial, 1984). This is consistent with the CD18 requirement for each stimulus. That is, C5a is associated with Gram-positive pneumonia, and both of these stimuli elicit CD18-independent migration. Conversely, C5a is not as critical for PMN responses to Gram-negative bacterial pneumonia, which is CD18-dependent.

**5. Immune-Complex Deposition.** Immune-complex (IgG) deposition injury in lungs is induced in rats by administering BSA i.v. and anti-BSA antibodies (IgG) intratracheally. Immune complexes form at the vascular-airway interface and serve as the focus for inflammatory responses, which include airway PMN recruitment and vascular leak (Johnson et al., 1984). PMN emigration is due to airway generation of CINC-1, MIP-2, and C5a (Shanley et al., 1997). In addition, production of IL-1, TNF- $\alpha$ , and PAF contribute to both PMN emigration and vascular leak (Mulligan and Ward, 1992; Warren, 1992). Blocking either PMN influx or oxygen-derived free radicals, which are generated from activated PMNs or alveolar macrophages, protects from edema and vascular leak (Mulligan et al., 1992b).

Airway PMN emigration in response to IgG immune complexes relies on multiple integrins. Antibodies to CD11a/CD18, CD11b/CD18, or  $\beta_1$ - (CD29) integrins block PMN emigration to various degrees (Mulligan et al., 1993, 1995). Furthermore, the antibody to CD11b/CD18 is more effective when administered intratracheally than i.v., whereas antibodies to the other integrins have the opposite relationship. The mechanism for the differential inhibition is not known.

**6. Summary and Research Needs.** The pulmonary circulation is unique in comparison to the systemic circulation in that there are large numbers of margined PMNs, and migration preferentially occurs from capillaries. Moreover, the involvement of adhesion molecules varies with the stimulus. For example, the involvement of CD18 during PMN emigration is stimulus-dependent, and the degree of dependence is likely determined by the particular cytokines, chemokines, and other chemoattractants produced in the airways in response to each stimulus. Studies to test this hypothesis adequately have not been performed. In addition, the mechanism of

CD18-independent PMN emigration has yet to be characterized.

Because of the different inflammatory responses elicited by various airway stimuli, it is important that animal models exist that represent the unique clinical scenarios that occur in humans. Many models are limited in usefulness insofar as they only accurately describe PMN responses specific to a particular stimulus. For example, even though LPS and lipoteichoic acid can be used to model the PMN migration during Gram-negative and Gram-positive infections, respectively, there are likely important differences in inflammatory responses to bacterial products compared with the whole bacterial organism. Similarly, the contralateral injury to untreated lung lobes in acid aspiration models is not observed in other models of PMN-mediated lung injury, so that the applicability of this model to other forms of PMN-mediated injury is open to question. It is also important to consider PMNs emigrating into airways from the bronchial circulation in addition to PMNs arising from the pulmonary capillaries. Airway instillation of stimuli via the trachea would presumably induce inflammation along airway walls containing vessels of the bronchial circulation as well as reach the deeper lung and pulmonary capillary beds. Conversely, models that use intralobar instillation may deliver inflammatory stimuli anatomically closer to alveolar regions and may therefore induce relatively more PMN emigration from the pulmonary vasculature compared with the bronchial circulation. Airway stimuli that have CD18-dependent or -independent components might have different influences on PMN emigration from bronchial and pulmonary circulations. There is a need to develop animal models that will encompass these various nuances.

#### *E. Polymorphonuclear Leukocyte Migration during Endotoxemia: A Special Case of CD18 Inhibition?*

Blood-borne endotoxin (i.e., endotoxemia) inhibits PMN migration to Gram-negative bacterial airway stimuli (Nelson et al., 1990; Frevert et al., 1994; Wagner et al., 1996). More recently, it has been shown that endotoxemia inhibits PMN migration to a variety of airway stimuli and that inhibition is selective for stimuli that require CD18 (Wagner et al., 1999). The mechanism(s) of inhibition is unknown, but it may be related to the failure of circulating PMNs to express CD18 in a spatial and temporal manner that is consistent with competent TEM processes.

CD18 expression on PMNs in the pulmonary circulation is not significantly up-regulated when a CD18-dependent stimulus is present in airways (Burns and Doerschuk, 1994). By contrast, CD18 is up-regulated on PMNs before they adhere and migrate toward an airway stimulus that does not require CD18. In vitro, endotoxin induces cytoskeletal changes, loss of L-selectin, and the expression of CD18 on isolated PMNs (Lynn et al., 1991), and similar changes are described on PMNs isolated

from endotoxemic animals (Frevert et al., 1994). Thus, early CD18 up-regulation by blood-borne endotoxin might preclude competent CD18-dependent migration, but it may be a normal PMN response in the process of CD18-independent migration. We have proposed that an untimely expression of CD18 on circulating PMNs may violate the stepwise sequence of rolling, firm adhesion, and diapedesis, resulting in aborted or dysregulated interactions between PMNs and ECs (Wagner et al., 1999). Similar inhibitory effects on PMN chemotaxis by exposure to endotoxin have been described in vitro (Bignold et al., 1991).

#### IV. Pharmacologic Intervention

Interfering with the ability of inflammatory cells to adhere to and migrate through endothelium during certain disease states can be beneficial. Indeed, one of the many ameliorative effects of glucocorticoid or nonsteroidal anti-inflammatory drug (NSAID) treatment is to limit the activation and recruitment of leukocytes. As our understanding of the specific processes that drive TEM increases, so will our ability to design pharmaceuticals that are more specific and that have fewer side effects than generally acting anti-inflammatory agents. For example, antagonistic ligands based on the active sites of integrins and selectins are being developed that are effective in several animal models of inflammation and autoimmune disease. In addition, the pathways of signal transduction associated with the activation of adhesion molecules have been proposed as targets for the design of a new class of anti-inflammatory agents. Several novel and some more conventional therapies are discussed briefly below (Table 3).

##### A. Lipid A Analogs

Endotoxin itself can bind specifically to CD18 integrins on PMNs, where it may interfere with ligand binding or modulate intracellular pathways of activation (Flaherty et al., 1997; Ingalls et al., 1998). Endotoxin inhibits pulmonary PMN migration at doses that do not cause overt organ injury in rats (Wagner et al., 1999). Thus, it may be possible to design an analog of endotoxin that is without inflammasogenic properties but that re-

tains the suppressive effects on PMN migration. In this regard, synthetic products that are structural modifications of lipid A, the active component of the LPS molecule, have been used both as antagonists to endotoxin-cell interactions and as bioactive, endotoxin-mimicking agents. One such compound, monophosphoryl lipid A, protects from ischemic injury to cardiac myocytes by inducing nitric oxide production without activating acute inflammatory responses (Gyorgy et al., 1999). In another model of cardiac ischemia, injury to vascular ECs is inhibited by monophosphoryl lipid A by a nitric oxide-independent mechanism (Richard et al., 1999). LPSs vary in their effects in mammalian systems, and furthermore, they can antagonize each other's effects presumably via differences in their lipid A reactivity with cellular targets. An endotoxin from *Escherichia coli* has recently been characterized that can selectively activate monocytes (Hone et al., 1998). Specifically, this strain of endotoxin elicits production of chemokines but not TNF- $\alpha$  or IL-1, and furthermore, it can antagonize TNF- $\alpha$  and IL-1 production induced by more inflammasogenic endotoxins. A synthetic analog (E5531) based on the structure of a nontoxic lipid A from *Rhodobacter capsulatus* is effective at blocking TNF- $\alpha$  production and lethality in endotoxemic mice and inhibits endotoxin-induced responses in human monocytes (Kawata et al., 1995, 1999). Understanding the mechanisms underlying the diverse activities of lipid A analogs and endotoxins will provide insight into using these compounds as core structures from which to develop novel anti-inflammatory compounds that may provide selective modulation of inflammation processes.

##### B. Cytokine Blockers

Blockade of proximal cytokines produced early in inflammatory cascades would hypothetically block distal injurious events including leukocyte adhesion, migration, and activation. This premise led to the development and clinical evaluation of antibodies and receptor-based antagonists for TNF- $\alpha$ , IL-1, and PAF among others, for use in subjects with systemic inflammation. Although effective at blocking their target cytokines, treatment with blockers of proximal cytokines have resulted in little improvement and occasionally have worsened mortality in patients with sepsis and systemic inflammatory response syndromes (reviewed in Cohen, 1995; Zeni et al., 1997; Baue et al., 1998). Sepsis and related inflammatory syndromes are characterized by the sequential production of inflammatory mediators and of opposing anti-inflammatory immunosuppressive factors, both of which may be required for resolution of inflammation and return to homeostasis (Wagner and Roth, 1999). Lack of efficacy of cytokine blockers might arise from either the lack of production of opposing cytokines or an imbalance in pro- and anti-inflammatory mediators (Bone, 1996). That is, cytokines such as TNF- $\alpha$  and IL-1 may be required to initiate anti-inflamm-

TABLE 3  
Therapeutic intervention and targets

Inflammatory Target	Proposed Therapies
Cytokine production, activity	Receptor antagonists, antibodies, steroids, NSAIDs $^2$ , lipocortin nonapeptides, LPS analogs
Chemokine production, activity	Receptor antagonists, antibodies, steroids, NSAIDs $^2$
Rolling, tethering of PMNs	Fucoidan, glycomimetics
Firm adhesion	Leumedins, ligand-based peptide inhibitors, NSAIDs, HMG-CoA reductase inhibitors, nitric oxide donors, LPS analogs $^2$
Diapedesis	Antiproteases $^2$ , ligand-based peptides $^2$

matory pathways required for proper resolution of inflammation.

Systemic inflammatory responses that occur during sepsis or shock are special cases of "toxic inflammation" with complex and as yet incompletely defined mechanisms. Thus, therapies that target TNF- $\alpha$ , IL-1, or PAF may be more efficacious at ameliorating simpler and more localized inflammatory conditions as psoriasis, arthritis, and minor infections. Conversely, blocking the effects of a more distal mediator such as IL-8 might better regulate inflammation without disrupting the balance of pro- and anti-inflammatory cytokines. Antibodies directed against IL-8 block neutrophil-mediated tissue injury in animal models of endotoxemia and lung reperfusion injury (Sekido et al., 1993; Yokoi et al., 1997). A panel of human anti-IL-8 antibodies has been recently characterized in rabbits and monkeys and is proposed for use in clinical trials (Yang et al., 1999b). By binding only soluble IL-8 and not IL-8 on the surfaces of neutrophils, ECs, or erythrocytes, these antibodies avoid the side effects accompanying complement-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity. Similarly designed antibodies directed against monocyte or eosinophil chemokines might someday provide therapies for inflammatory conditions based on the involvement of specific cell types.

#### C. Steroids

Effects of glucocorticoids are pancellular, limiting both the production of inflammatory mediators by leukocytes and the response to those mediators by target cells (e.g., other leukocytes, endothelial and parenchymal cells). Studies *in vitro* demonstrate a direct effect of glucocorticoids on cultured ECs and isolated PMNs to inhibit expression of adhesion molecules in response to LPS or PAF (Cronstein et al., 1992; Filep et al., 1997). Similar observations are made after glucocorticoid treatment during cardiopulmonary bypass in humans and in endotoxemic rats (Hill et al., 1994; Davenport et al., 1998b). However, it is not clear if inhibition is due to a direct effect of steroids on PMNs and ECs as observed *in vitro* or if steroids modify the production of a secondary mediator(s). For example, partial inhibition by dexamethasone of pulmonary PMN migration in response to airway endotoxin is associated with decreased production of TNF- $\alpha$  and IL-1 in airways, but the chemokine MIP-2 is not significantly affected (O'Leary et al., 1996; Yi et al., 1996). Some chemokines, such as LIX, are sensitive to glucocorticoid regulation, whereas CINC and MIP-2 are not (Rovai et al., 1998). Clearly, steroids can affect multiple sites of the inflammatory cascade and likely modify adhesive and chemotactic processes by multiple mechanisms.

Glucocorticoids inhibit inflammatory responses in part by modulating the function of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Wissink et al., 1998). Activation of NF- $\kappa$ B is associated with the regulation of a battery of genes that

code for cytokines and other inflammatory mediators, and it also regulates the expression of endothelial and leukocytic adhesion molecules (Lee et al., 1996; Brostjan et al., 1997). Another adhesion-inhibitory mediator of glucocorticoids is lipocortin-1, a potent endogenous inhibitor of phospholipase A<sub>2</sub> (Flower and Rothwell, 1994). Dexamethasone-induced inhibition of leukocyte-EC adhesion is reversed by antibodies to lipocortin (Mancuso et al., 1995), suggesting that lipocortin can negatively regulate adhesion processes. Furthermore, several studies demonstrate that treatment of PMNs with lipocortin or a peptide analog can inhibit chemotaxis and emigration induced by a variety of stimuli both *in vitro* and *in vivo* (reviewed by Perretti, 1998; Zouki et al., 2000). Thus, at least two potential mechanisms exist that might explain the direct effect of steroids on leukocyte and endothelial responses during inflammation.

#### D. Nonsteroidal Anti-Inflammatory Drugs

Cyclooxygenase (COX) mediates the conversion of arachidonic acid to prostaglandins, thromboxanes, and other lipid mediators involved in inflammation and normal physiologic function (reviewed in DuBois et al., 1998). The anti-inflammatory effects of nonsteroidal anti-inflammatory drugs (NSAIDs) are most often attributed to their ability to inhibit the inducible form of COX (i.e., COX-2). The constitutive form of the enzyme, COX-1, is required for normal function of platelets, the kidney, and the lining of the gastrointestinal tract. Depending on the cell type, the activity of both enzymes leads to the production of prostanoids and other lipid mediators. Therefore, NSAIDs that inhibit both COX-1 and COX-2 (e.g., aspirin) can result in both a diminution of inflammation and side effects of compromised platelet, kidney, and gastrointestinal functions.

Inhibition of adhesive and migratory processes between leukocytes and ECs can occur after treatment *in vitro* with NSAIDs such as aspirin (Pierce et al., 1996; Pillinger et al., 1998), ibuprofen (Kapiotis et al., 1996), and oxicams (Garcia-Vicuna et al., 1997). This suggests that COX metabolites mediate TEM in some models. By contrast, some prostanoids block these processes. For example, adhesion and TEM *in vitro* can be inhibited by prostaglandin (PG) E<sub>1</sub>, PGE<sub>2</sub>, or iloprost, a prostacyclin (PGI<sub>2</sub>) analog (Riva et al., 1990; Bath et al., 1991; Oppenheimer-Marks et al., 1994; Lou et al., 1998). These observations suggest that NSAIDs may inhibit migratory processes by a mechanism unrelated to their effect on COX activity and prostanoid production. This is best illustrated by the ability of sodium salicylate, a weak COX inhibitor to block CD18 activation and adhesion of PMNs, whereas indomethacin, a potent COX inhibitor, is ineffective at modulating adhesion in the same *in vitro* model (Gerli et al., 1998; Pillinger et al., 1998). Two hypotheses for the effects of salicylate on adhesion are 1) it inhibits mitogen-activated kinase Erk, which is required for CD18 activation in neutrophils (Pillinger et

al., 1998), and 2) it inhibits NF- $\kappa$ B activation by blocking the phosphorylation and degradation of inhibitor- $\kappa$ B (Pierce et al., 1996). Ibuprofen has also recently been characterized as an inhibitor of NF- $\kappa$ B activation by a similar mechanism to that described with salicylates (Stuhrmeier et al., 1999). The mechanism of adhesion blockade by members of the oxicam family of NSAIDs has not been fully determined. Taken together, inhibition of Erk and NF- $\kappa$ B pathways by NSAIDs might represent critical anti-inflammatory actions, especially with regard to recruitment of inflammatory cells, that are equally important as the COX-inhibitory action of these compounds.

#### E. Anti-Selectin Therapies

1. *Fucoidan.* Fucoidan is a homopolymer of a sulfated fucose product of marine algae that has structural similarities to saccharide residues on endogenous ligands for L- and P-selectins. Fucoidan blocks selectin binding in vitro and has been used as an effective tool to inhibit leukocyte emigration in a variety of pulmonary disease and injury models (Mizgerd et al., 1996; Shimaoka et al., 1996). However, the polyanionic nature of fucoidan also imparts heparin-like antithrombogenic properties (Giroux et al., 1998). Furthermore, fucoidan inhibits the ability of endotoxin to elicit plasminogen activator inhibitor release from ECs (Soeda et al., 1995) and blocks chemokine-induced activation of monocytes (Heinzelmann et al., 1998). Thus, although fucoidan inhibits leukocyte rolling (Kuebler et al., 1997; Yamaguchi et al., 1997) and pulmonary PMN recruitment (Mizgerd et al., 1996), its protective effect in animal models might be due to other actions on inflammatory pathways. Efforts to synthesize fucose-containing glycosides might yield agents that are more specific for selectin inhibition (Koenig et al., 1997; Sakagami et al., 1999).

2. *Glycomimetics.* In addition to sulfated sugar groups, saccharide units with sialic acids are a critical moiety on selectin ligands. One such nonsulfated sialylated saccharide is sialyl-Lewis<sub>x</sub> (SLE<sub>x</sub>), soluble forms of which protect from selectin-dependent lung and liver injury (Mulligan et al., 1993; Rubio-Avilla et al., 1997; Hayashi et al., 1999). Extensive work with a range of lectin compounds suggest that sulfation, sialylation, and the number and type of saccharide units contribute to the selectivity and affinity for selectin binding (Shailubhai et al., 1997; Varki, 1997; Weitz-Schmidt et al., 1999). For example, a sulfated SLE<sub>x</sub> glycopeptide based on the NH<sub>2</sub> terminus of PSGL-1 is more effective at blocking P-selectin-dependent adhesion than free SLE<sub>x</sub> (Leppanen et al., 1999). The effectiveness of sulfated SLE<sub>x</sub> has been demonstrated in animal models of smoke inhalation and ischemia-reperfusion injury in lungs (Reignier et al., 1997; Tasaki et al., 1998). Mulligan and coworkers (Mulligan et al., 1998) synthesized a series of nonsaccharide sulfated sialyl compounds that protect from PMN-dependent lung injury. Thus, sulfation and sialylation appear to be the most critical structural determinants of these classes of glycomimetics.

Newer generations of glycomimetics have other structural modifications. Compounds based on the polycyclic molecule glycyrrhizin, a natural product from licorice, contain neither sulfate nor sialyl groups but can nonetheless inhibit leukocyte binding to ECs in vitro (Kim et al., 1998) and prevent PMN-dependent ischemic injury in vivo (Kilgore et al., 1998). In addition, combinatorial chemistry methodologies have yielded oligonucleotides that bind specifically to P-selectin (Jenison et al., 1998). This is in contrast to most other glycomimetics (i.e., fucoidan, SLE<sub>x</sub>), which bind two or all three selectins, or for which selectin preference has not been fully examined. This information is important when determining therapies that limit inflammation but not specific immune system or repair processes.

#### F. Anti-Integrin Therapies

1. *Leumedins.* Leumedins are a class of small molecular-weight pharmaceuticals that inhibit CD18-dependent PMN adhesion (Endemann et al., 1996, 1997). One of the better characterized leumedins, NPC 15669 (*N*-[9H-{2,7-dimethylfluoren-9-methoxy}-carbonyl]-L-leucine), is effective at blocking pulmonary leukocyte recruitment during ventilator-associated injury (Rimensberger et al., 1998), bacterial pneumonia (Jorens et al., 1994), and allergic airway responses (Kaneko et al., 1994; Agusti et al., 1998). The mechanism of leumedin-induced inhibition of integrin function is not completely understood. Although leumedins can inhibit the expression of CD18 in response to stimuli (Bator et al., 1992), the mechanism of adhesion blockade by leumedins is due neither to inhibition of CD18 expression from granular stores nor to the inactivation of CD18 already in the cell membrane (Endemann et al., 1996). NPC 15669 also inhibits responses to the chemoattractants fMLP and C5a, but it inhibits receptor binding only of fMLP (Smith et al., 1995). In addition, leukocyte responses such as superoxide production in response to downstream activators of G-protein pathways are also inhibited by NPC 15669, suggesting that leumedins act at both receptor and postreceptor levels. These and other studies (Bennett et al., 1993; Navab et al., 1993) provide evidence that the anti-inflammatory effects of leumedins are not specific to integrin-mediated adhesion.

2. *Ligand-Based Peptide Inhibitors.* Cells that express  $\beta_1$ - and  $\beta_3$ -integrins can adhere to extracellular matrix components, such as collagen and fibronectin among others, via interaction with specific peptide sequences [arginine-glycine-aspartic acid (RGD)] contained in these matrix ligands. Disintegrins are low-molecular-weight, cysteine-rich peptides that contain RGD sequences and occur naturally in snake venoms and sperm proteins. By interfering with RGD-integrin interactions, disintegrins can inhibit  $\beta_1$ - or  $\beta_3$ -mediated adhesion of platelets, melanoma cells, and ECs to matrix

components in vitro (Niewiarowski et al., 1994; Juliano et al., 1996; Staiano et al., 1997).

Little information has been published on the ability of disintegrins to modulate  $\beta_2$ -dependent responses of leukocytes in animal or isolated cell systems. However, incubation of PMNs with RGD-containing molecules can inhibit PMN chemotaxis to IL-8 or fibrin degradation products (Gudewicz et al., 1994; Gross et al., 1997). By comparison, an antibody to Mac-1 did not affect PMN chemotaxis to IL-8 but did modulate responses to fibrin degradation products (Gross et al., 1997). These two findings suggest that  $\beta_2$ - (e.g., Mac-1) and RGD pathways represent different chemotactic responses of leukocytes to inflammatory stimuli.

A unique,  $\beta_3$ -like RGD-responsive receptor found on monocytes and PMNs, called the leukocyte response integrin (LRI), has been characterized as a modulator of various leukocyte functions such as phagocytosis and adhesion (Gresham et al., 1992; Carreño et al., 1993). Antibodies to LRI can block RGD-dependent adhesion and chemotaxis to the basement membrane component, entactin (Senior et al., 1992). Peptides containing RGD can indirectly interfere, presumably via the LRI, with Mac-1 binding to immobilized complement protein C3bi (Van Strijp et al., 1993). Therefore, it is possible that  $\beta_2$ -dependent, RGD-sensitive pathways of inflammation might be targeted by treatment with disintegrins and thereby diminish leukocytic adhesion, chemotaxis, and phagocytosis. To date, this hypothesis has not been adequately tested.

Neutrophil inhibitory factor (NIF) is a glycoprotein from canine hookworm that inhibits Mac-1-dependent PMN adhesion to ECs (Muchowski et al., 1994). NIF binds to the I domain of the  $\alpha$ -chain of Mac-1 (i.e.,  $\alpha_M$ ; CD11b) but does not interact with the I domain in the  $\alpha$ -chains of LFA-1 or p150,95 ( $\alpha_L$  and  $\alpha_X$ , respectively) (Zhang and Plow, 1997). The I domain of integrin  $\alpha$ -chains contain critical peptide sequences that provide the specificity for binding to ligands such as fibrinogen and C3bi. As such, NIF represents a prototype inhibitor of integrin-mediated PMN adhesion that is highly specific for a selected integrin. The peptide sequences of the I domain of CD11b that are required for NIF binding have been determined (Zhang and Plow, 1997); however, the complementary sequences and structural requirements of NIF have yet to be elucidated. Understanding the mechanisms of NIF interactions with the I domain will add insight into possible anti-adhesive compounds directed against the I domain on other  $\alpha$ -chains. Development of these as well as agents directed against RGD-binding motifs will allow for selective inhibition of PMN adhesion based on integrin specificity for the targeted ligand.

#### *G. 3-Hydroxy-3-methyl-glutaryl Coenzyme A Reductase Inhibitors*

Two classes of drugs prescribed for heart and vascular disease, 3-hydroxy-3-methyl-glutaryl coenzyme A

(HMG-CoA) reductase inhibitors and nitric oxide-donating compounds, have been documented in laboratory animals to inhibit recruitment or activation of neutrophils in vivo (Dunzendorfer et al., 1997; Wallace et al., 1997; Tang et al., 1998). The unexpected side effects of these compounds have generated interest in their development as specific adhesion-blocking therapeutics.

Lovastatin, pravastatin, and simvastatin are examples of HMG-CoA reductase inhibitors referred to as "statins". These interfere with cholesterol synthesis and are effective at lowering serum cholesterol in hypocholesterolemic individuals. Suppressed PMN responses in patients receiving statin therapy are associated with lower PMN membrane cholesterol levels (Day et al., 1997). Studies in vitro show that statins inhibit PMN adhesion to ECs by lowering the expression of LFA-1 and Mac-1 on leukocytes (Niwa et al., 1996; Weber et al., 1997, 1999). At least one report suggests that a mechanism of integrin inhibition may involve the direct binding of statins to the I domain on the  $\alpha_L$ -subunit of LFA-1 (Kallen et al., 1999).

Another mechanism of statin-induced inhibition may be its effect on G-proteins that are involved in integrin activation. HMG-CoA reductase inhibitors are used to block targeting of Ras and Rho families of GTPase proteins to their active sites in the cell membrane by inhibiting protein prenylation (Cuthbert and Lipsky, 1997; Ghosh et al., 1999). Activation of Ras and Rho are required in some in vitro models of cell adhesion (Zhang et al., 1996; Laudanna et al., 1996, 1997). Thus, inhibition of the mobilization of certain G-proteins to sites in the membrane might negatively affect integrin activation to some stimuli. This has been demonstrated in isolated lymphocytes, in which Rho-induced adhesion is inhibited by lovastatin (Zhang et al., 1999). However, mobilization of GTPases mediates both activation and inhibition of integrins because H-Ras inhibits integrin binding, whereas R-Ras activates integrins (Zhang et al., 1996; Hughes et al., 1997; Sethi et al., 1999). Clearly, further investigation is needed to explain how statins modulate G-protein function and whether intervention in these pathways by statins is an effective therapeutic approach for controlling PMN adhesion.

#### *H. Nitric Oxide Donors*

Nitric oxide-donating agents promote vasodilation by activating cGMP in endothelial and smooth muscle cells and provide effective therapy for angina and hypertension-associated symptoms that occur during cardiovascular disease. Administration of nitric oxide or of nitric oxide-donating species inhibits neutrophil adhesion in animal models of ischemia/reperfusion, lung injury, and peritonitis (Wallace et al., 1997; Fukatsu et al., 1998; Grisham et al., 1998; Sato et al., 1999). Furthermore, animals with low basal nitric oxide release have greater basal PMN adherence compared with that in normal animals (Lefer and Ma, 1993; Lefer et al., 1999). Similar

effects have not been reported in humans. However, exposure of isolated human or rat PMNs to nitric oxide inhibits their adhesion in vitro and is associated with increased intracellular cGMP and reduced CD18 expression on PMNs (Banick et al., 1997; Ohashi et al., 1997; Chello et al., 1998; Kosonen et al., 1999). These effects might be related to ADP ribosylation of actin in PMNs that occurs after exposure to nitric oxide (Clancy et al., 1995). In contrast to PMNs, incubation of EC monolayers with nitric oxide donors induces P-selectin- and PAF-dependent PMN adhesion (Okayama et al., 1999). The difference in responses of ECs and PMNs in vitro and the effects on PMN adhesion in whole animal models might be due to the local concentration of nitric oxide.

Neither HMG-CoA reductase inhibitors nor nitric oxide donors have been used therapeutically to inhibit PMN adhesion in humans. However, when more completely characterized, their unique mechanisms of action may suggest novel designs for drugs that are more specific to PMN adhesion and less active at their present cellular targets (i.e., cholesterol synthesis and vascular tone).

### V. Summary

Expansion of our understanding of the processes involved in inflammatory cell recruitment has revealed new molecular targets for pharmacologic intervention. Although several pharmacologic manipulations of the adhesion and migratory processes have been successful in animal models, further development and refinement of anti-adhesive agents are necessary before they are likely to be maximally effective in humans. At least three primary knowledge gaps need to be bridged to assess the safety and benefits of the potential therapies discussed above: 1) specificity of action, 2) matching the appropriate therapy to the target disease, and 3) effects of pharmacological intervention on the mounting of normal and beneficial inflammatory responses.

Selectins and integrins have structural similarities within their respective families that present the potential for cross-reactivity of anti-adhesive agents directed against similar residues or structures. First, ligand-based pharmaceuticals that take advantage of the structural dissimilarities in integrin subunits or in selectin glycosylation or sulfation patterns can be designed only after the binding sites and the secondary and tertiary structures of these molecules are better defined. Second, the specific adhesion molecules responsible for cell recruitment need to be defined better for each particular disease state or inflammatory scenario before therapies can be effective without producing untoward side effects. The potential diversity and specificity for adhesion molecule involvement is illustrated in detail above for the special case of pulmonary PMN migration, during which adhesion molecule involvement depends on the airway stimulus and the qualitative nature of inflammatory

mediators. Last, the consequence of interrupting the balance between pro-inflammatory and anti-inflammatory mediators to the normal resolution of inflammation needs to be assessed for each therapeutic strategy. For example, the benefits of controlling inflammation by limiting neutrophil mobilization must be weighed against the costs of immunosuppression or the contribution of neutrophils to tissue repair. From continued research efforts in these areas will emerge novel pharmaceuticals that are effective in preventing and/or treating inflammatory diseases.

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